TITLE INVESTIGATION OF THE BASIS OF SPECIFIC SECRETION OF CELLULASE CELV BY THE OUT APPARATUS OF ERWINIA CAROTOVORA SUBSPECIES CAROTOVORA.

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Investigation of the basis of specific secretion of cellulase CelV by the Out apparatus of *Erwinia carotovora* subspecies *carotovora*.

by

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To Mum and Dad

CONTENTS

SUMMARY

Erwinia species cause soft rot in a range of economically important plants. The secretion of cellulases and pectinases, via the Out apparatus is considered a major pathogenic determinant. The Out proteins are encoded by a cluster of at least 13 genes, *outC* to *outO*, and show homology to the pullulanase secretion proteins of *Klebsiella oxytoca*, and secretion proteins of other plant and animal pathogens.

This study concerns the nature of the interaction between the Out apparatus and the secreted enzymes, with a view to identification of the recognition motif(s) on the secreted proteins that allow specific secretion.

Secretion deficient mutants were isolated using hydroxylamine and PCR mutagenesis. These were characterised with respect to activity and stability of the CelV derivatives; ability to secrete other proteins; and DNA sequence. Two classes of mutant were identifed. The first class exhibited accumulation of a catalytically active but conformationally defective product which did not affect the secretion of other proteins. These were therefore predicted to be defective at the level of recognition. The second class exhibited accumulation of a stable, full size product which interfered with the secretion of pectate lyase and were therefore predicted to be blocked within the translocation apparatus. The isolation of intragenic suppressors of some of these mutants is described.

Several deleted derivatives of CelV were constructed, all of which were severely affected in their ability to be secreted, even when only-12 carboxy terminal amino acids were affected.

In conclusion, recognition for secretion is highly dependent on conformational integrity of both of the functional domains of which the enzyme is composed. The pathways followed by cellulase and pectinases appear to be initially separate, converging to a common translocation apparatus.

In addition, cellulase, although having limited independent macerating ability, was identified as playing a vital role in the collaborative action of exoenzymes during attack of potato tubers.

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[1] The page number refers to the page preceeding the table.

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself, except that sequencing of the *out* cluster was carried out in collaboration with other members of the research group of Prof. George Salmond. All sources of information are acknowledged by means of reference.

Some of the work presented in this thesis has been included in the following publications:-

Reeves, P.J. *et al* (1993) Molecular cloning and characterisation of 13 *out* genes from *Erwinia carotovora* subspecies *carotovora*: genes encoding members of a general secretory pathway,(GSP) widespread in Gram negative bacteria. Molecular Microbiology 8:443-456.

Walker, D.S. *et al* (1994) The major secreted cellulase, CelV of *Erwinia carotovora* subspecies *carotovora* is an important soft rot virulence factor. Molecular Plant-Microbe Interactions, in press.

ABBREVIATIONS

ADP = adenosine diphosphate

APS = ammonium persulphate

ATP = adenosine triphosphate

CBD = cellulose binding domain

Cel = cellulase

CMC = carboxymethyl cellulose

DDA = double Difco agar

DNA = deoxyribonucleic acid

Eca = *Erwinia carotovora* subspecies *atroseptica*

Ecc = *Erwinia carotovora* subspecies *carotovora*

Ech = *Erwinia chrysanthemi*

EDTA = ethylenediaminotetraacetic acid

EMS = methane sulphonic acid ethyl ester

HCA = hydrophobic cluster analysis

IPTG = isopropyl-*B*-galactopyranoside

LBA = Luria broth agar

NBA = nutrient broth agar

OBR-cellulose = Ostazin Brilliant Red cellulose

Peh = polygalacturonase

Pel = pectate lyase

pfu = plaque-forming units

Prt = protease

SDS = sodium dodecyl (lauryl) sulphate

Taq = *Thermus aquaticus* DNA polymerase

TEMED = N, N, N', N' -tetramethylethylenediamine

Tris = Tris (hydroxymethyl) aminoethane

ts = temperature sensitive

UV = ultraviolet light

v/v = vloume/volume

w/v = weight/volume

CHAPTER 1

INTRODUCTION

1.1 Introduction to *Erwinia*

The soft rot erwinias are enterobacterial pathogens which exert their effect via maceration of host tissue, and affect a wide range of economically important plants. The group comprises two main species, *Erwinia chrysanthemi* (*Ech*) and *Erwinia carotovora*, the second being divided into three subspecies, *carotovora* (*Ecc*), *atroseptica* (*Eca*), and *betavasculorum*. They are capable of exerting their effect in the field or in storage, and besides soft rot induce various other symptoms on the growing plant. *Ech* is the causative agent of carnation wilt and chrysanthemum stem canker; while *Eca* can cause Blackleg on potato, the main symptom of which is leaf wilt.

Ecc is an aetiological agent of soft rot in stored crops, potatoes and carrots in particular (Perombelon and Kelman 1980), and as such presents a considerable economic problem. The rate and success of infection is affected by factors such as oxygen potential, temperature and humidity, but these are obviously not always easy, or indeed economical, to control, so that attention has turned towards the major pathogenicity determinant, the synthesis of an arsenal of extracellular macerating enzymes. The synthesis and secretion of extracellular enzymes has been extensively studied in both *Ecc* and *Ech*.

1.2 Introduction to cellulases

Cellulose and hemicelluloses (including xylan) together account for greater than 50% of plant biomass, making them the most abundant terrestrial organic molecules. The significance of cellulases and hemicellulases in the renewal of this energy source is therefore evident. Cellulases are classified according to the degradative forms which they hydrolyse, as figure 1.1 shows. Internal glycosidic bonds are cleaved by endoglucanase, followed by the synergistic attack of endo- and exo-glucanases. Final hydrolysis of the resulting oligosaccharides is effected by *B*-glucosidase.

In recent years, genes encoding as many as 100 cellulases and xylanases, fungal and prokaryotic, have been sequenced (Gilkes *et al.*, 1991a). Analysis of the amino acid sequences, combined with hydrophobic cluster analysis (HCA), a technique which reveals similarities in apparent secondary structure between proteins of very low sequence identity even despite variable intervening sequences (Henrissat *et al.*, 1989), has led to the identification of evolutionary relationships between enzymes from diverse organisms. In particular, it has resulted in the identification of the modular nature in which cellulases are made up of varying combinations of functionally autonomous domains.

In general, cellulases have a single catalytic domain, classified by HCA into nine families (table 1.1). Many of these families comprise cellulases from diverse organisms,

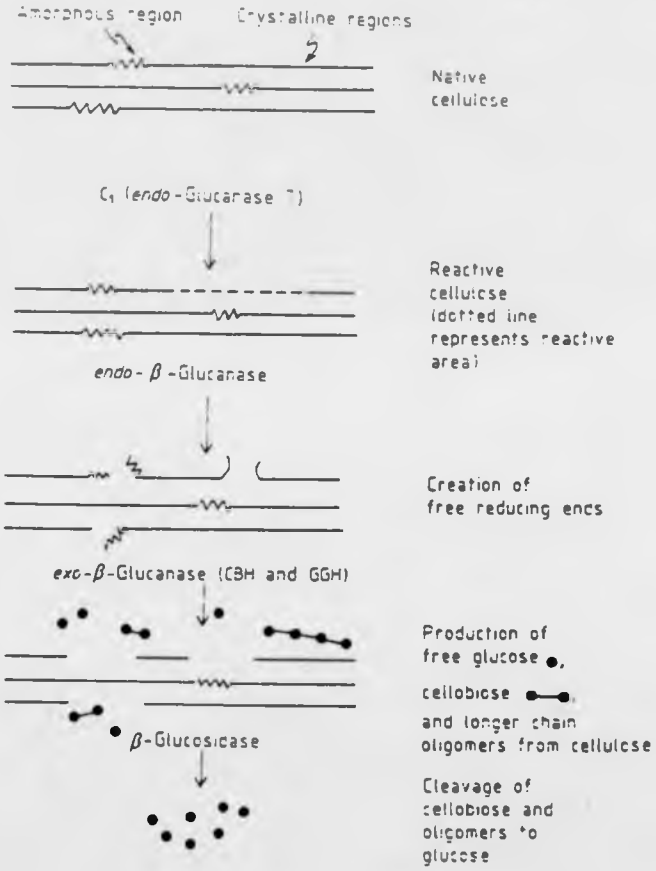

Figure 1.1. Enzymes involved in the cellulose degradation pathway.

Taken from Kosaric *et al* (1983).

Family	Organism	Enzyme	No. of amino acids	Catalytic domain[a]	
				Terminus	No. of amino acids
A					
1	*Bacillus* sp. strain 1139	EgI	770	N	185
2	*Bacillus* sp. strain KSM-635	EgI	912		≤444
3	*Bacillus* sp. strain N-4 gene pNK1	CelB	665	N	307
4	*Bacillus* sp. strain N-4 gene pNK2	CelA	384	N	305
5	*Bacillus* sp. strain N-4 gene pNK3	CelC	>800	N	~350
6	*Bacillus lautus*	CelB	536		
7	*Bacillus polymyxa*	EgI	369		
8	*Bacillus subtilis* N-24	EgI	463		
9	*Bacteroides ruminicola*	EgI	>363	N	
10	*Butyrivibrio fibrisolvens* A46	CelA	396		
11	*Butyrivibrio fibrisolvens* H17c	EndI	921	N	185
12	*Caldocellum saccharolyticum*	CelB[b]	1,011	C	308
13	*Clostridium acetobutylicum*	EgI	409	N	~300
14	*Clostridium cellulolyticum*	CelA	449	N	~300
15	*Clostridium thermocellum*	CelB	928	N	~409
16	*Clostridium thermocellum*	CelC	322		
17	*Clostridium thermocellum*	CelF	780	N	340
18	*Clostridium thermocellum*	CelH	~860	C	~410
19	*Erwinia chrysanthemi*	CelZ	385	N	305
20	*Fibrobacter succinogenes*	EgI[b]	635	C	~416
21	*Rhizobium* sp. strain Y 20	EgI	375		
22	*Ruminococcus albus* F-40	EgII	363		
23	*Ruminococcus albus* SY1	CelA	~365		
24	*Ruminococcus albus* SY1	CelB	~385		
25	*Trichoderma reesei*	EgIII	397	C	327
26	*Xanthomonas campestris*	EngXCA	468	N	~350
B					
1	*Cellulomonas fimi*	CenA	418	C	284
2	*Microbispora bispora*	CelA	426	N	~280
3	*Streptomyces* sp. strain KSM-9	CasA	318		
4	*Trichoderma reesei*	CbhII	447	C	185
C					
1	*Humicola grisea*	CbhI	506		
2	*Phanerochaete chrysosporium*	CbhI	~494	N	~425
3	*Trichoderma reesei*	CbhI	~494	N	425
4	*Trichoderma reesei*	EgII	431	N	363
5	*Trichoderma viride*	Cbh	496	N	439
D					
1	*Bacillus circulans*	Bgx	~378		
2	*Cellulomonas uda*	EgI	336		
3	*Clostridium thermocellum*	CelA	445	N	~384
4	*Erwinia chrysanthemi*	CelY			
E					
1	*Butyrivibrio fibrisolvens*	CedI	947		
2	*Cellulomonas fimi*	CenB	1,012	N	607
3	*Cellulomonas fimi*	CenC	1,069	Internal	489
4	*Clostridium thermocellum*	CelD	608	N	>543
5	*Clostridium stercorarium*	CelZ	981	N	474
6	*Ruminococcus flavefaciens*	SGSP270-6	705	N	~450
7	*Persea americana*	EgI	484		
8	*Persea americana*	CelI	484		
9	*Persea americana*	CelJ			
10	*Pseudomonas fluorescens* subsp. *cellulosa*	EgI	930		
F					
1	*Bacillus* sp. strain C-125	XynA	396		
2	*Butyrivibrio fibrisolvens*	XynA	378	N	~350
3	*Caldocellum saccharolyticum*	CelB[b]	1,011	N	347
4	*Caldocellum saccharolyticum*	XynA	312		
5	*Caldocellum saccharolyticum*	ORF-4	312		
6	*Cellulomonas fimi*	Ces	443	N	315
7	*Clostridium thermocellum*	XynZ	809	C	~350
8	*Crepidotus albidus*	Xyn	311		
9	*Pseudomonas fluorescens* subsp. *cellulosa*	XynA	585	C	345
10	*Pseudomonas fluorescens* subsp. *cellulosa*	XynB	555	C	272
11	*Thermomonas aurantiacus*	Xyn	269		
G					
1	*Bacillus circulans*	Xyn	185		
2	*Bacillus pumilis*	XynA	201		
3	*Bacillus subtilis*	Xyn	182		
4	*Clostridium acetobutylicum*	XynB	234		
H					
1	*Aspergillus aculeatus*	Egl	237		
2	*Erwinia carotovora*	CelS	232		
I					
1	*Ruminococcus flavefaciens*	CelA	552		

[a] Deduced from positions of putative linkers, sequence comparison, and truncation experiments
[b] Catalytic domain 1 (family F, no. 1) catalytic domain 2 (family A, no. 12) of the bifunctional cellulase of *Caldocellum saccharolyticum*

Table 1.1. Families of cellulase and xylanase catalytic domain.

The table shows enzymes from all 9 families identified to date, on the basis of hydrophobic cluster analysis. The table shows, from left to right, the family (A to I) and number designated to each; the organism; the name of the enzyme; the total size in amino acids; the position of the catalytic domain (C = carboxy terminal, N = amino terminal) within the protein; and the number of residues in the catalytic domain. Taken from Gilkes *et al* (1991a).

with not only Gram positives appearing in the same family with Gram negatives, but also fungal with bacterial, and prokaryotic with plant enzymes.

A number of different types of non-catalytic domains have been identified. Cellulose binding domains (CBDs) are often found, in various positions in the protein, and in various numbers. They are usually around 100 residues in length, and often share certain cysteines, tryptophans, glycines and asparagines (Gilbert and Hazlewood 1993). CBDs have also been classified by HCA into several families. Table 1.2 shows examples in each of these from enzymes in the catalytic family A. As this table shows, these domains do not necessarily have demonstrated cellulose binding properties, and indeed they vary considerably in strength and specificity of binding. This and figure 1.2 also illustrate that classification of CBDs is completely independent of that of catalytic domains.

Regions of repeated sequence are a further class of auxillary domain. These possess highly variable length and number of tandem repeats, and even domain duplication. Finally, of particular importance to the modular structure of cellulases is the existence of short linker sequences of extended secondary structure. These vary from 6 (CenC of *Cellulomonas fimi*) to 118 residues (SGSP of *Dictyostelium discoidium*) and the majority are rich in either serine or a combination of proline and threonine. These regions of extended structure function as spacers, allowing independent folding of the domains which they link, and it has been

CBD	CBD	Examples			
Family	Length	Enzyme	Organism	Binding?	Position
I	35-36aa	EG3	*Trichoderma reesei*	+	N
II	86-148aa	EG3	*Trichoderma reesei*		I
III	131-144aa	End	*Bacillus subtilis* DLG		C
		End	*Bacillus subtilis* N-24		C
		CelZ	*Clostridium stercorarium*	+	I
		CelZ	*Clostridium stercorarium*		C
		CelV	*Ecc*	+	C
IV	240aa	CelE	*Clostridium thermocellum*	+	I
V	61aa	EGZ	*Ech*	+	C

Table 1.2. Families of cellulose binding domain.

The table shows examples of enzymes from the 5 families of cellulose binding domain (CBD), all of which are in family A with regard to their catalytic domain. The table shows, from left to right, the family (I to V); the length of the CBD; the name of the enzyme; the organism; whether or not cellulose binding activity has been demonstrated (+ indicates demonstrated binding activity); and the position of the CBD in the enzyme (C = carboxy terminal, N = amino terminal, I = internal). Based on Coutinho *et al.* (1992), Beguin (1990) and Gilkes 4et al. (1991a).

suggested (e.g. Gilbert and Hazlewood 1993) that from an evolutionary point of view they play a role similar to that of introns, in that they allow the shuffling of functional domains without damage to important coding sequences. This, along with domain duplication and gene transfer, explains how such a wide range of cellulases, composed of different combinations of similar domains, evolved. Some idea of this shuffling effect is given by the examples given in figure 1.2.

Linker regions are susceptible to proteolysis, a property that has been widely used in various systems to illustrate the independent actions of the functional domains (e.g. Py *et al.*, 1991b; Gilkes *et al.*, 1988), catalytic or cellulose binding properties of the individual domains often being very similar to those of the whole protein, as is also the case when domains from different cellulases are mixed and matched (e.g. Poole *et al.*, *1991).* A study by Ferreira *et al.*, (1990) indicated that variation of the linker region of a xylanase of *Pseudomonas fluorescens* subspecies *cellulosa* also had little effect on catalytic or cellulose binding properties.

1.3 The cellulases of *Erwinia*

Both *Ech* and *Ecc* produce one major *B*-1,4-endoglucanase, EGZ (Guiseppi *et al.*, 1988) and CelV (Cooper and Salmond 1993), respectively, which both account for over 95% of activity seen. They both possess Family A catalytic domains at the N-terminus, and are secreted with over 95% efficiency

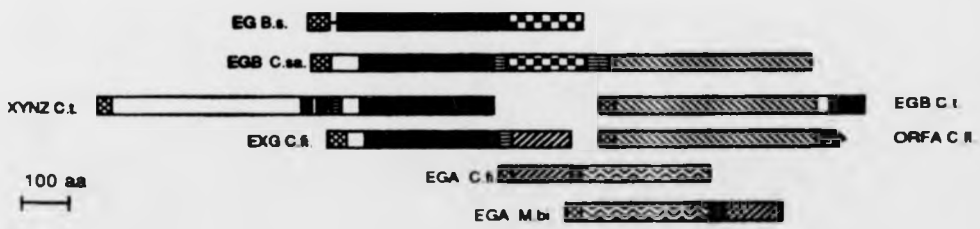

Figure 1.2. Domains of extended similarity between some cellulases and xylanases.

The diagram shows EG of *Bacillus subtilis*, EGB of *Caldocellum saccharolyticum*, XYNZ and EGB of *Clostridium thermocellum*, EXG and ORFA of *Cellulomonas flavigena*, EGA of *Cellulomonas fimi*, and EGA of *Microbispora bispora*. Domains showing significant similarity are filled with the same pattern. Taken from Beguin (1990).

(*Py et al.*, 1991a). In addition, both species produce an additional endoglucanase, CelY (Family D, Guiseppi *et al.*, 1991) of *Ech*, and CelS (Family H, Saarilahti *et al.*, 1990b) of *Ecc*. These are expressed at very low levels in laboratory conditions, although they could be plant-induced.

1.4 Other extracellular enzymes

The principle determinant of maceration and therefore virulence is the secretion of several pectate lyase (Pel) isozymes (Hinton *et al.*, 1989). *Ech* EC16 produces 4 isozymes, arranged in two clusters (*pelA,E* and *pelB,C*. Barras *et al.*, 1987; Tamaki *et al.*, 1988), while *Ech* 3937 produces five, in clusters ADE and BC (Kotoujansky *et al.*, 1985). Those within a cluster are more closely related, suggesting a duplication event was involved in their evolution. Ecc also produces four (Hinton *et al.*, 1989; Zink and Chatterjee 1985), which are considerably diverged from those of *Ech*.

Other extracellular enzymes involved in various stages of the pectin degradation pathway have been identified, such as pectin lyase (Pnl) (Chatterjee *et al.*, 1990), pectin methyl-esterase (Pme) (Heikinheimo *et al.*, 1991, Kotoujansky *et al.*, 1985), and exo- and endo-polygalacturonase (Peh) (Hinton *et al.*, 1990, Saarilahti *et al.*, 1990a, He and Collmer 1990). Figure 1.3 shows the degradation pathway of pectin and galacturonate in *Ech*, illustrating the role of extracellular pectinases at the start of this cascade of reactions. In *Ech*, many of the other genes encoding

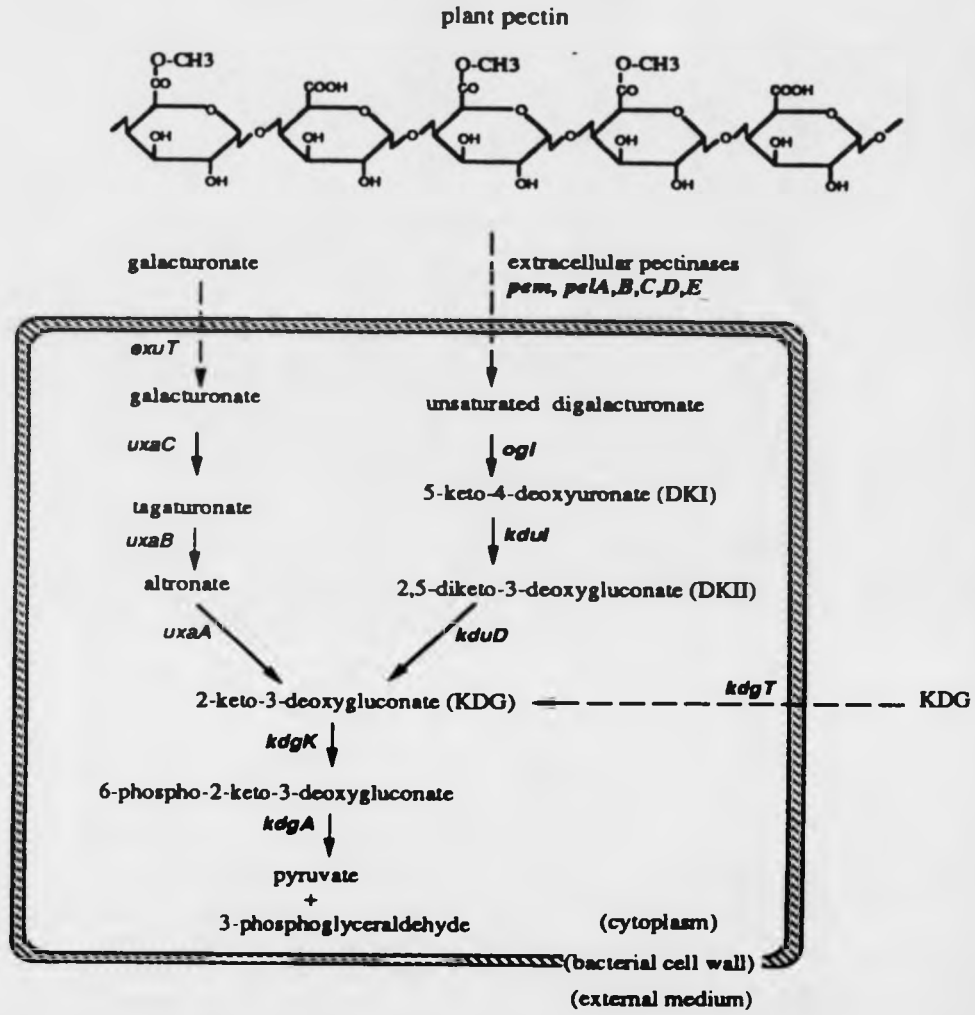

Figure 1.3. The galacturonate and pectin degradation pathway of *Erwinia chrysanthemi*.

The genes whose products catalyse the various steps are given next to the arrows. *pem* encodes pectin methyl esterase; *pelA-E*, pectate lyases; *ogl*, oligogalacturonate lyase; *kdul*, 5-keto-4-deoxy uronate isomerase; *kduD*, 2-keto-3-deoxygluconate oxidoreductase; *uxaC*, uronate isomerase; *uxaB*, altronate oxidoreductase; *uxaA*, altronate hydrolase; *kdgK*, 2-keto-3-deoxy gluconate kinase; kdgA, 2-keto-3-deoxy-6-phosphogluconate aldolase. *exuT* and *kdgT* encode transport systems mediating entry of galacturonate and 2-keto-3-deoxy gluconate, respectively, into the cells. Taken from Reverchon *et al* (1991).

components of this pathway have also been cloned (*ogl*, *kduI*, *kduD*, *kdgT*, *kdgK*, *kdgA*). Expression of these, as well as *pelA-E* and *pem* is inducible in the presence of polygalacturonate or galacturonate (the effect being due to the degradative derivatives of these, KDG, DKI and DKII, Condemine *et al.*, 1986). This effect is mediated by the KdgR product, which negatively regulates all the genes concerned (Reverchon *et al.*, 1990). Sequence comparison of several genes controlled by KdgR has revealed a highly conserved 25 base pair imperfect palindrome (e.g. Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992; Condemine and Robert-Baudouy 1991), shown by reporter gene studies (Reverchon *et al.*, 1991) and gel retardation (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992; Nasser *et al.*, 1992) to be the binding site for KdgR. In addition, Condemine *et al.*, (1992) have shown that there are also several so-called "KdgR boxes" in the *out* cluster of *Ech*, encoding the secretory apparatus responsible for the translocation of Pels (and Cel) across the outer membrane. The result is that a whole range of genes involved in various ways in the degradation of pectin and galacturonate can be "switched on" by the presence of substrate, which has obvious advantages to the cell in terms of efficient channelling of resources.

Both *Ecc* and *Ech* also produce several metalloproteases (Barras *et al.*, 1986; Kyostio *et al.*, 1991; Delepelaire and Wandersman 1989). Those of *Ech* have been studied in detail, particularly with regard to secretion, as discussed later. They are synthesised as inactive zymogens, which undergo

processing to an active form only once the external milieu is reached (Delepelaire and Wandersman 1989), a mechanism which serves to protect the producing cell.

1.5 Why study *Erwinia*?

In addition to being economically important, *Ecc* offers several other advantages to the researcher. Firstly, it is closely related to *E. coli*, thereby benefiting from the long standing importance of this organism in the world of genetics. Secondly, mainly as a result of this relationship, it is genetically amenable, many techniques having been directly adapted from those developed in *E. coli*. For example, transformation (Hinton *et al.*, 1985), generalised transduction (Chatterjee and Brown 1980; Toth *et al.*, 1993), conjugation mapping with Hfr and R-prime strains (Chatterjee *et al.*, 1985) and gene cloning technology (e.g. Barras *et al.*, 1984; Kotoujansky *et al.*, 1985) have all been easily adapted, it is a host to *E. coli* replicons, and can be transduced using bacteriophage lambda and derivatives (Hinton and Salmond 1987) when carrying the *E. coli lamB* gene (Salmond *et al.*, 1986).

1.6 Introduction to secretion

The secretion of extracellular proteins plays a key role in the interactions of cells or organisms with their environment and with other cells or organisms. Gram negative bacteria secrete relatively few, but these proteins (e.g. cholera toxin of *Vibrio cholerae*; polysaccharidases of *Erwinia* and *Xanthomonas* species) are often primary

determinants of virulence in both plant and animal hosts.

The Gram negative cell boundary represents a formidable challenge to any secretion mechanism. Proteins must be translocated across both the inner and outer membranes, from one hydrophilic environment (the cytoplasm) to another (the external medium) without becoming trapped in the periplasm. This requires not only the crossing of two hydrophobic barriers (by a largely hydrophilic protein), but also of the highly complex periplasmic space, including the rigid peptidoglycan layer, and is therefore a formidable task. Figure 1.4 illustrates the complexity of this barrier.

As figure 1.5 illustrates, mechanisms by which Gram negative bacteria secrete proteins can be divided into two groups, according to whether or not the protein enters the periplasm as it crosses the envelope, and genetic analysis of *Ecc* and *Ech* has shown that both pathways are utilised (Salmond and Reeves 1993; Wandersman 1992, for reviews). In the two-step mechanism, the initial step involves recognition of a typical N-terminal signal sequence by the general export system (of which the best studied example is the Sec machinery of *Escherichia coli*) and translocation of the protein across the cytoplasmic membrane, resulting in the cleavage of the signal sequence. The second step then requires specific factors for translocation across the outer membrane. This is the mechanism by which pectinases and cellulases are secreted by *Erwinia* species. The one-step mechanism, on the other hand, does not require recognition

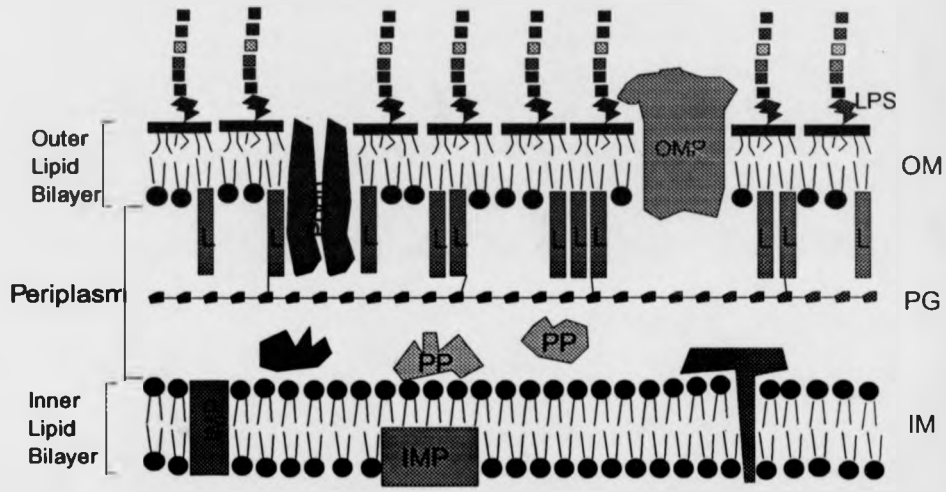

Figure 1.4. Illustration of the complexity of the Gram negative cell wall.

Abbreviations: OM = outer membrane, IM = inner membrane, PG = peptidoglycan, L = lipoprotein, LPS = lipopolysaccharide, PP = periplasmic protein, IMP = inner membrane protein, OMP = outer membrane protein. Adapted from a diagram by G.P.C. Salmond.

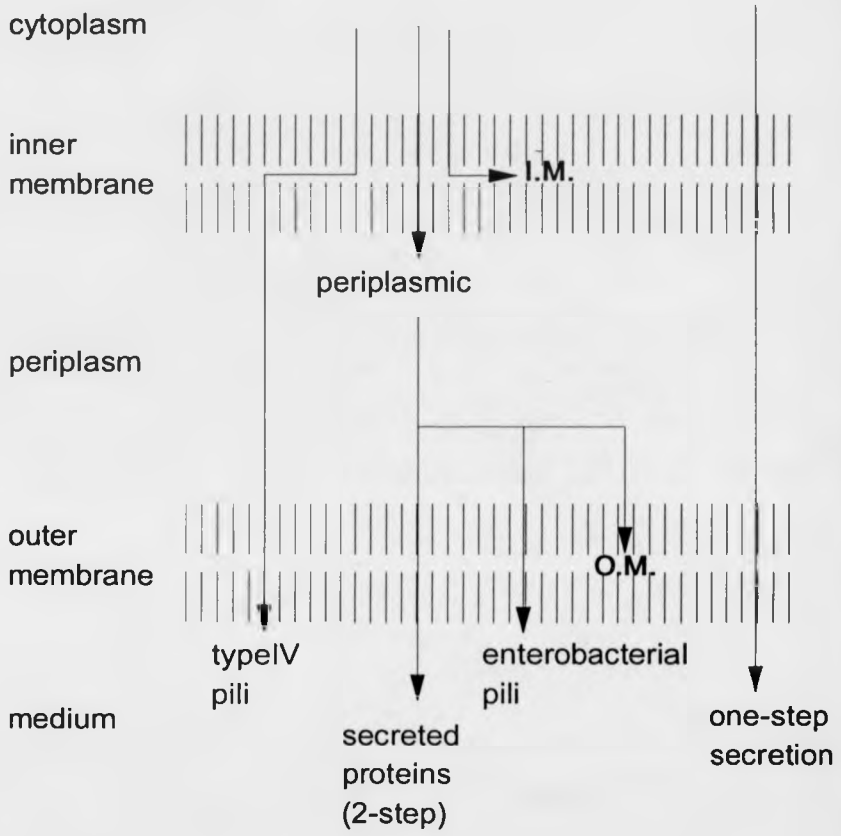

Figure 1.5. Translocation pathways across the Gram negative cell envelope.

The diagram includes the one- and two-step secretion pathways, and assembly of proteins into the inner and outer membranes. IM = inner membrane, OM = outer membrane.

or cleavage of a classical signal sequence, although it does appear to be directed by a C-terminal secretion signal, as discussed later. The secreted protein traverses both membranes at once, via a channel formed by several accessory proteins. The best characterised example is alpha-haemolysin secretion by *E. coli*, but a highly homologous system is responsible for protease secretion by *Erwinia* species.

One digression from the classification of secretion mechanisms into 1-step and 2-step is the existence of several proteins which are Sec-dependent in their passage across the inner membrane, but require no other factors for their translocation across the outer membrane. These autosecretory proteins include the *E. coli* enterotoxins, ST_A (Yang *et al.*, 1992) and ST_B (Drefus *et al.*, 1992).

1.7 Haemolysin secretion: a one-step mechanism

The haemolysin secreted by uropathogenic strains of *E. coli* is translocated into the medium by a mechanism which does not require a classical N-terminal signal sequence, is independent of the general export (Sec) pathway (Mackman *et al.*, 1987) and in which the secreted protein does not stop in the periplasm (Mackman *et al.*, 1986). Instead, secretion is directed by two specific proteins, HlyB and HlyD, both inserted into the inner membrane, together with the outer membrane protein TolC (Wandersman and Delepelaire 1990).

The metalloproteases of Ech are also secreted by a one-step mechanism (Barras *et al.*, 1986), being synthesised without a

signal sequence. Both are synthesised as inactive zymogens, activation involving cleavage of an N-terminal "pro-sequence", a post-secretion event. The protease genes are found in an operon along with homologues of *hlyB (prtD)*, *hlyD (prtE)* and *tolC (prtF)*, as well as *inh*, encoding a periplasmic inhibitor, a safety mechanism for the producing cell (Delepelaire and Wandersman 1989; Letoffe *et al.*, 1990). A similar operon is responsible for secretion of alkaline protease by *Pseudomonas aeruginosa* (Duong *et al.*, 1992), encoding *Apr* homologues of PrtD, E and F, as well as Inh.

Homologues of HlyB and D have also been reported in the secretion of various members of the haemolysin family (cyclolysin of *Bordetella pertussis*, leukotoxin of *Pasteurella haemolytica*, (Letoffe *et al.*, 1990), haemolysin of *Vibrio cholerae*, (Alm and Manning 1990)), as well as the antimicrobial peptide, subtilin of *Bacillus subtilis* (Chung *et al* 1992), protease of *Serratia marcescens*, the nodulation protein NodO of *Rhizobium leguminosarum* (Scheu *et al., 1992), and the antibiotic syringomycin of Pseudomonas syringae* pv. *syringae* (Quigley *et al.*, 1993).

1.7.1 Assigning roles to the Hly proteins and homologues

Topological studies combining immunolocalisation; protease sensitivity (Delepelaire and Wandersman 1991); and analysis of PhoA and Bla fusions (Wang et al., 1991; Schulein *et al.*, 1992) indicate that HlyB and D (and their homologues) span the inner membrane, HlyB with six transmembrane segments and

HlyD with one, although depending on the method used there is also evidence for association with the outer membrane, suggesting that they form part of a complex that spans both membranes (Blight and Holland 1990). Sequence homology (Blight and Holland 1990) indicates that the large carboxy-terminal cytoplasmic domain of HlyB contains two nucleotide binding sites (Higgins *et al.*, 1986), probably the source of energy for the translocation process. Indeed, recent investigations (Koronakis *et al.*, 1993) have demonstrated that HlyB has ATPase activity, that it shows affinity for both ATP and ADP, and that binding of either results in a significant conformational change in the soluble domain. Figure 1.6 shows a recent model based on these observations.

1.7.2 The search for a secretion signal

Mutational analysis and sequence comparisons (Koronakis *et al.*, 1989; Stanley *et al.*, 1991; Kenny *et al.*, 1992) indicated that the secretion signal is located in the N-terminal region and highlighted the possible importance of three secondary structural features: an amphipathic helix, a cluster of charged residues, and a weakly hydrophobic terminal sequence. This has been supported by various studies in which progressively shorter C-terminal fragments were fused to reporter genes (Kenny *et al.*, 1991; Delepelaire and Wandersman 1990; Letoffe and Wandersman 1992). These studies identified a short sequence of 40-50 amino acids at the extreme C-terminus, capable of directing secretion. In many of the fusions made, however, a much larger section, encompassing a glycine-rich repeat motif, is

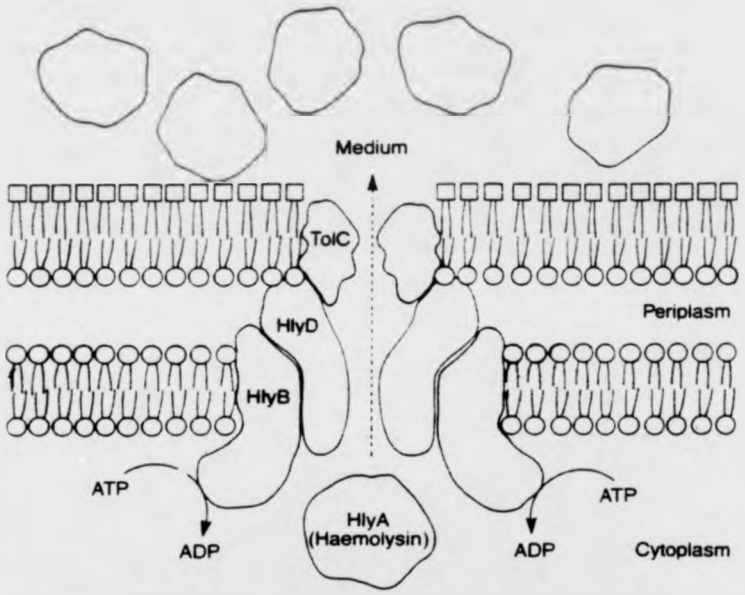

Figure 1.6. Model of the haemolysin secretion apparatus of *E. coli*.

As the diagram illustrates, HlyB and HlyD corractionate with the inner membrane, but are also found in outer membrane fractions, suggesting that they form a pore complex that spans both membranes. TolC is an outer membrane protein. From Reeves and Salmond (1992).

necessary, perhaps reflecting the importance of maintaining an independently folded signal motif separate from the "passenger" domain. The signal seems to be quite tolerant of mutation: a single missense mutation completely destroying secretion has not yet been identified (Kenny *et al.*, 1992).

1.8 The general secretory pathway

The universal step of the general secretory pathway (GSP) (following which proteins follow diverse paths, such as secretion, location in the periplasm, or assembly in either membrane, as figure 1.5 illustrates) constitutes the first step in the two step secretion pathway, translocation across the outer membrane relying on factors discussed in detail later. Much of the work concerning the GSP has been in *E. coli*, where its most significant role is translocation of periplasmic and membrane proteins, *E. coli* being a very poor secretor. Nevertheless, given the genetic amenability and global significance of *E. coli*, it provides a useful model in which to study the mechanisms and interactions involved, and from which to extrapolate.

1.8.1 The Sec apparatus

A minimum of six proteins seem to be required for targeting and translocation across the cytoplasmic membrane. SecA is a peripheral membrane protein with ATPase activity (Lill *et al.*, 1990); SecB is a cytoplasmic antifolding factor (in other words, a molecular chaperone); SecD, E, F and Y are integral inner membrane proteins. In addition, two inner membrane signal peptidases have been identified, LspI (LepB)

Component	Mr (KDa)	Function	Prl Phenotype	Location
SecA	104	ATPase	PrlD	peripheral IM
SecB	18	Chaperone		cytoplasm
SecD	67	Translocase		integral IM
SecE	14	Translocase	PrlG	integral IM
SecF	39	Translocase		integral IM
SecY	39	Translocase	PrlA	integral IM
LepB	36	Signal peptidase		integral IM
LspA	18	Signal peptidase		integral IM
Ppp	25	Signal peptidase		integral IM

Table 1.3. Components of the *E. coli* export machinery.

The table shows, from left to right, the name of the protein (PPP = prepilin peptidase, Lep = leader peptidase, Lsp = lipoprotein signal peptidase); its size; predicted function; the Prl phenotype, where these have been isolated; and its predicted location (IM = inner membrane). Based on Pugsley (1993), Bieker-Brady and Silhavy (1992) and Schatz and Beckwith (1990).

and LspII (LspA) (Reviewed by Schatz and Beckwith 1990).

SecY, with SecE is sufficient to allow SecA- dependent translocation of precursor proteins in reconstituted systems (Akimanu *et al.*, 1991). Their central role is confirmed by the fact that only temperature sensitive or reduced functionality mutants have been identified, true nonsense mutants being lethal. SecY is an integral membrane protein, with 10 trans-membrane segments (Akiyama and Ito 1987). Mutations have been identified which result in either defective translocation of precursors (Baba *et al.*, 1990; Ito *et al.*, 1989) or suppress certain defects in the signal sequence of exported proteins (known as Prl mutants) (Sako and Iino 1988). Since these mutants have been found in various regions of the *secY* gene, and since temperature sensitive mutants at their permissive temperature are highly susceptible to blockage by non-exportable proteins or high level expression of exportable proteins (Ito *et al.*, 1989), it perhaps could be suggested that it forms part of the translocation channel. This is supported by the fact that antibodies to various regions of the protein all have an inhibitory effect (Tokuda *et al.*, 1990); and by a recent cross-linking experiment (Joly and Wickner 1993). This experiment used a preprotein/ dihydrofolate reductase fusion (which jams in the membrane, due to the folded structure of the latter domain), combined with standard cysteine-based cross-linking methods to show the close involvement of SecY and SecA in translocation. Sequence comparison of various SecY homologues (Tschauder *et al.*,

1992; Suh *et al.*, 1990) indicates a high degree of conservation in the transmembrane segments and in the cytosolic loops between transmembrane segments 4 and 5, and 8 and 9. Since mutants isolated to date do not affect these conserved residues, it seems that they are directly involved in function, since mutations altering important amino acids, structurally or functionally, would be lethal. A dominant negative mutant of SecY has been isolated (Shimaike *et al.*, 1992), which when plasmid-borne severely inhibits export by wild type cells. The mutation consists of a 3 amino acid deletion between transmembrane segment 5 and cytoplasmic domain 5. The authors suggest that the mutant could be catalytically deficient, but retaining the ability to associate with other components to form a complex, the dominant effect being exerted by competition with wild type SecY for a place in this complex.

SecE is essential for growth, and Prl mutants (that suppress signal sequence defects) have been identified, indicating a central role. It consists of three transmembrane segments, but only the C-terminal one, plus surrounding regions, seem to be required for function (Schatz *et al.*, 1991).

Both SecE and SecY have been studied using a particularly ingenious method called suppressor-directed inactivation (SDI, Bieker and Silhavy, 1990; Bieker-Brady and Silhavy 1992). This utilises a LamB-LacZ fusion, the signal sequence of LamB directing the large C-terminal LacZ fragment to the secretion machinery, where is causes lethal jamming. This is a secretion-specific jamming effect, defective signal

sequences resulting in benign cytoplasmic accumulation of the hybrid. However, when a PrI suppressor is used, the fusion is targetted to this, and the PrI component sequestered, resulting in a Mal-sensitive phenotype (since maltose induces overexpression of the hybrid). This phenotype can be complemented by the wild type allele, since the wild type does not recognise the mutated signal sequence. In PrlA/SecY strains, the hybrid jams in a transmembrane manner, resulting in an inactive translocation complex; while in PrlG/SecE strains it jams prior to signal sequence processing, i.e. pretranslocation. The authors used these complexes to look at titration of various components. Using a ts mutant at semi-permissive temperature, expression of the component under study will be limiting. Involvement in the pre-translocation or translocation complex will therefore titrate the limited amount available and increase maltose sensitivity, while non-involvement will not. This approach has been used to identify SecY, D and F as components of the translocation complex; and SecA and E as involved in both pretranslocation and translocation itself.

SecA is the only other component for which PrI mutants have been isolated, and is the third and final component of the reconstituted system mentioned earlier. It is required for translocation, but not for binding of the pre-protein to the membrane (Cunningham *et al.*, 1989), and has a high affinity for membranes, and in particular SecE and Y (Hartl *et al.*, 1992; see cascade model later). ATPase activity is stimulated by acidic lipids, and stabilised by interaction

with precursor protein (Lill *et al.*, 1990).

SecD and F are the only constituents that are expressed from an operon (Gardel *et al.*, 1990). Both have large periplasmic domains, null mutants are not lethal, and Prl mutants have not been isolated. These factors suggest an auxillary role, in the final stages of export (Gardel *et al.*, 1990). Antibody inactivation of SecD has indeed been shown to inhibit the release of maltose binding protein from the membrane, which results in trypsin sensitivity, as opposed to the trypsin resistance which the released form exhibits (Matsuyama *et al.*, 1993).

1.8.2 SecB: a chaperone

SecB acts as a chaperone, in that it is a soluble factor required for maintenance of the prefolded (loose) conformation that is required for translocation competence (reviewed by Kumamoto 1991). SecB mutants show reduced translocation efficiency, apparently relying on the low basal expression of heat shock proteins, which have been identified as able to substitute for SecB function (Altman *et al.*, 1991). When synthesised in the absence of SecB, translocation competent preprotein (e.g. prePhoE, deCock and Tommassen 1992) is still produced, but this requires the addition of SecB for translocation into inner membrane vesicles. Concerning the role of SecB in maintenance of the translocation-competent conformation the situation is unclear; it seems that the degree of importance in this respect varies depending on the preprotein studied (deCock and Tommassen 1992; MacIntyre *et*

al., 1991).

1.8.3 The signal sequence

The classical amino-terminal signal sequences required to direct export by the Sec apparatus lack direct sequence homology, but do possess a series of conserved motifs: an amino-terminal positive region; a hydrophobic core of around 10 residues; 6 to 8 amino acids with a high turn-former (e.g. proline) content; and a cleavage site consisting of AXA^ (where X is any residue, and ^ is the site of cleavage) (Landry and Gierasch 1991). The targetfor recognition by the secretory apparatus seems not to be contiguous, but rather, general features produced by these sequence motifs.

An important feature of the signal sequence is its independence of function, in that the nature of the mature component of the preprotein seems largely unimportant. Many cytoplasmic proteins can be exported simply by making them passenger of a signal sequence fused to the amino-terminal (e.g. MacIntyre *et al.*, 1991), and many proteins are unaffected in their ability to be exported, even by quite significant deletions at the carboxy-terminus or internally (e.g. Py *et al.*, 1993; Kornacker and Pugsley 1989; Huang and Schell 1992).

1.8.4 Formulating a complete model for export

Precisely how the signal sequence directs translocation is unclear. The basis of interaction with SecB is explained well by the Kinetic Partitioning Model of Hardy and Randall

(1992). Their *in vitro* studies showed an ability of SecB to distinguish between native-folded and non-native polypeptides, only binding to the latter. They suggest that the basis for this may be simply that the presence of the signal peptide, with its high hydrophobicity, retards folding, favouring association with SecB. Meanwhile proteins lacking a signal sequence will fold quickly and become inaccessible.

However, this is complicated by recent evidence (de Cock *et al.*, 1992), in which a series of mutants were constructed with internal deletions in PhoE. Except for two small mutants, all showed increased efficiency of translocation in the presence of SecB. Coimmunoprecipitation studies indicated that the mature and precursor forms showed similar affinity for SecB, while all of the deletions showed some affinity, albeit reduced. This puts a question mark on the proposed role of the signal sequence, and also points to the existence of multiple SecB binding sites. Given the diversity of proteins recognised by SecB, though, this is difficult to explain.

Concerning the remainder of the translocation process, Hartl *et al.*, (1990) have proposed a Cascade of Interactions model, which agrees with the data previously discussed, and with the major features of other models (e.g. Bieker-Brady and Silhavy 1992). This is based on affinity experiments using purified proOmpA, SecA and SecB, which indicated that SecB prevents non-productive low affinity association of pre-protein to membranes; SecB has direct affinity for SecA;

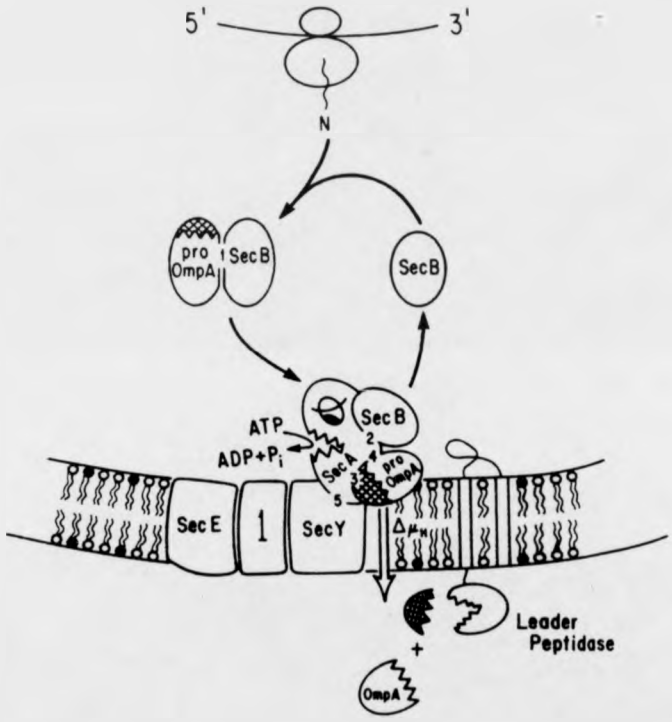

Figure 1.7. Model for the specific binding and membrane translocation of proOmpA.

The preprotein proOmpA forms a 1:1 complex with the chaperone SecB (1). High affinity interactions of SecB (2) and both the leader (3) and the mature (4) parts of proOmpA bind the proOmpA-SecB complex to SecA. SecA binds with high affinity to the SecY/E protein (5). ATP hydrolysis by SecA allows translocation of the precursor. The electrochemical potential increases the rate of translocation. Taken from Hartl *et al* (1990).

and that SecA is bound to the membrane at a high affinity receptor which includes SecY/E. Figure 1.7 shows the two-stage cascade of binding and release reactions proposed. SecB binds to the preprotein, and the complex then binds to SecA via SecB and the signal sequence. This complex then associates with the membrane-bound complex via high affinity interaction between SecA and SecY/E; and ATP hydrolysis by SecA drives translocation. The preprotein is thereby passed from the "targetting chaperone", SecB, to the "receptor chaperone", SecA. SecY and SecE would then, as already suggested, constitute the main translocatory "channel", with SecD and F playing an auxillary role on the periplasmic side of the membrane.

Cleavage of the signal sequence appears to occur very late in translocation, and appears to affect release from the membrane. Carlson and Silhavy (1993) have demonstrated that mutants of the outer membrane protein LamB that exhibit slow signal sequence cleavage have a profound effect on the cell because although the protein is largely in the periplasm and therefore attempts to form trimers in the outer membrane. it is nevertheless still associated with tne cytoplasmic membrane.

1.9 Out mutants and the Out cluster

Secretion mutants have been isolated using chemical mutagenesis (Reeves et al., 1993); bacteriophage mu derivatives; and transposons (Salmond *et al.*, 1986; Hinton and Salmond 1987; Hinton *et al.*, 1989). These have yielded

a class of mutants with the phenotype Cel-, Pel-, Prt+, when assayed on plates (Hinton and Salmond 1987), as a result of Pel and Cel activity being trapped in the periplasm, as shown by liquid culture assays of cell fractions. These mutants are known as Out mutants and define a group of genes encoding products therefore assumed to be required for the selective secretion of Pel and Cel across the outer membrane. This inability to secrete Pel and Cel results in reduced virulence (Murata *et al.*, 1990).

Cosmid complementation (identifying six complementation groups) and subcloning identified a 12.7Kb fragment, which, when sequenced, identified 13 open reading frames, designated *outC* to *outO* (Reeves *et al.*, 1993). This is a cluster of genes, all transcribed in the direction C to O, which may form an operon, since many of the open reading frames overlap slightly with their neighbours.

1.9.1 Homologies to the Out cluster

The *out* clusters of two *Ech* strains have been partially sequenced (Condemine *et al.*, 1992; He *et al.*, 1991; Lindberg and Collmer 1992) and are highly homologous to that of *Ecc*, but also exhibit several significant differences. Neither *Ech* EC16 (Lindberg and Collmer 1992) nor *Ech* 3937 (Kotoujansky, A. and coworkers, pers. com.) have a gene corresponding to *Ecc outN*, while *outO* is slightly larger, and appears to be transcribed separately from *outC-outM*. In addition, the situation upstream seems to be quite different: Condemine *et al.* (1992) reported that upstream of

Figure 1.8. Proteins related to the *out* cluster identified in other bacteria.

The gene clusters shown encode systems that perform a range of functions based on membrane translocation of macromolecules.

Boxes formed by dotted lines indicate that overall sequence identity is low, but relatively high in specific stretches: the C-terminal half of of OutD homologues; the signal sequences of OutG to J homologues; and the Walker boxes of OutE homologues.

To the left of the table, the names and organisms are given. To the right are given general functions of the gene clusters, while to the top predicted functions of particular proteins and their homologues are give; see text for more details.

Figures, where given, indicate percentage amino acid identity. = signifies that XCpA and PilD are identical.

Based on Reeves *et al* (1993) and Pugsley (1993).

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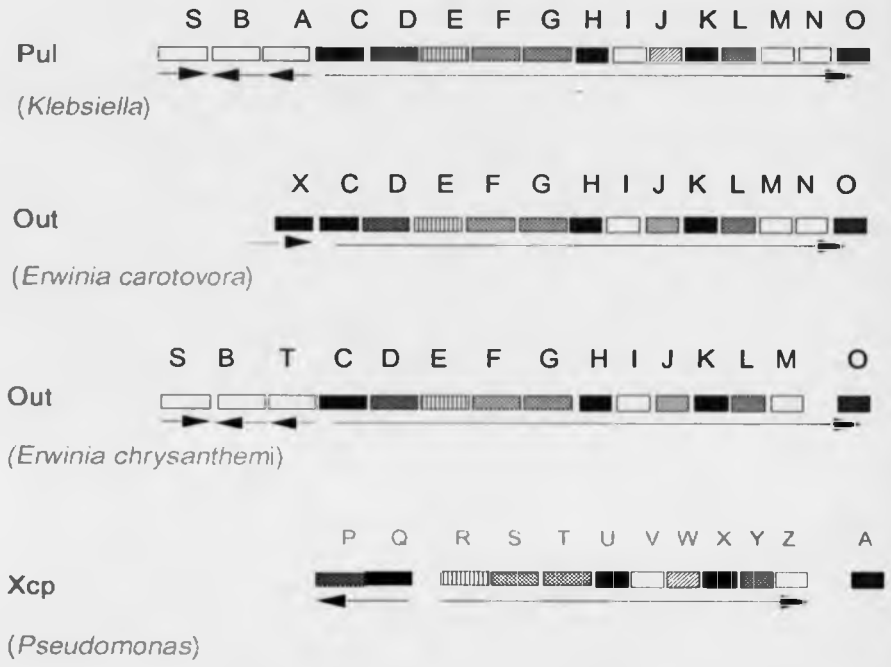


Figure 1.9. The organisation of secretion gene clusters.

Note that the Pul cluster and Ech Out cluster have a similar organisation upstream of *pulI/outC*; while Ech Out and Xcp differ from the others in that there is no *outN* homologue. The Xcp secretion genes are found in 3 separate loci. Based on Reeves *et al* (1993), Pugsley (1993), Condemine *et al* (1992), Bally *et al* (1991), Bally *et al* (1992), Akrim *et al* (1993).

Protein	Predicted size (KDa)	Hydrophobic regions
OutC	31.003	1
OutD	70.652	1
OutE	55.281	0
OutF	45.167	3
OutG	17.353	1
OutH	21.392	1
OutI	13.814	1
OutJ	24.853	1
OutK	36.573	1
OutL	47.644	1
OutM	18.722	1
OutN	26.775	1
OutO	31.135	4

Table 1.4. Characteristics of the Out proteins.

The table shows data taken from Reeves *et al* (1993). It shows, from left to right, the name of the Out protein, its predicted size in KDa, and the number of hydrophobic regions that are predicted to be either transmembrane stretches or membrane anchors.

outC in *Ech* 3937 there are three additional genes, *outT* (for which no homologues have been found in the data banks), *outB* (mutants of which have a leaky phenotype), and *outS* (which has an internal region of approximately 55 amino acids, of which over 50% are serine). Figure 1.9 shows a comparison of the upstream organisation in *Ech* 3937 and that of the pullulanase secretion cluster of *Klebsiella oxytoca*. In addition, two putative Kdg-binding boxes are found upstream of *outC* and *outT*, and indeed experiments using reporter gene fusions have shown that all except *outB* and *outS* are controlled by KdgR and induced by poly-galacturonate (Condemine *et al.*, 1992). Meanwhile, the *out* cluster of *Ech* EC16 is unique in being the only system, other than that of pullulanase secretion, discussed below, for which reconstitution of secretion (using a 40kb cosmid) has been achieved in *E. coli* (He *et al.*, 1991a).

The cluster of genes required for the secretion of pullulanase by *Klebsiella oxytoca* shows strong homology with the *Ecc out* cluster from *pulC* to *pulO* (d'Enfert *et al.*, 1989; Pugsley and Reyss 1990; Reyss and Pugsley 1990; Possot *et al.*, 1992). The cluster is expressed as an operon, with many of the open reading frames overlapping, as in the *out* cluster, and is induced by maltose via positive regulation by the MalT protein. In addition, it is clear that all the genes required have been identified since when these are expressed along with *pulA*, the structural gene, on an 18.8kb fragment, on pBR322, in the presence of maltose, secretion of pullulanase by *E. coli* K12 is exactly as seen in the native organism (d'Enfert *et al.*, 1987).

Similar clusters of secretion genes have been characterised in a number of other organisms. *Xanthomonas campestris*, the crucifer pathogen responsible for black rot, secretes endoglucanase, pectinases and protease, secretion of which is effected by the products of the *xps* genes. These appear to be arranged in two clusters, one carrying homologues of *out* E to J (Dums *et al.*, 1991); and the other an *out*D homologue plus two additional ORFs upstream for which there are no Out homologues (Hu *et al.*, 1992).

Aeromonas hydrophila secretes a wide range of extracellular enzymes including aerolysin, enterotoxin and protease, implicated as virulence factors. So far, only a 4.1kb fragment, capable of complementing a secretion deficient mutant, has been sequenced, identifying *exe* homologues to *out*E and F; and also parts of *out*D and G (Jiang and Howard 1992), suggesting that the secretion cluster could well be more extensive.

Pseudomonas aeruginosa is an opportunistic pathogen of various mammals, including humans. The Xcp products are responsible for secretion of exotoxin A, phospholipase C and elastase, important in the invasion of host tissue and protection from host defence systems. The *xcp* cluster (Filloux *et al.*, 1990; Bally *et al.*, 1991; Bally et al., 1992; Akrim *et al.*, 1993) comprises homologues to *outC* to *outM* and *outO*, but these differ in organisation. As figure 1.8 shows, xcp*A*, and *xcpP* and *Q* are not contiguous with the

bulk of the cluster; and the latter two genes are in the opposite orientation to their homologues.

Several *Pseudomonas aeruginosa* genes involved in the assembly of type IV fimbriae (or pili), another significant determinant of virulence, also exhibit homology to components of the *out* cluster. *pilA*, the structural gene encoding precursor subunits is expressed in an operon (controlled by PilR, S (Hobbs *et al.*, 1993) and T (Whitchurch *et al.*, 1990)) with *pilB*, *pilC* and pil*D*, all required in assembly (Nunn *et al.*, 1990). PilB is homologous to OutE, possessing consensus ATP binding sites and, like OutE, is predicted to be cytoplasmic (Nunn *et al.*, 1990), and may, with PilC, form a complex involved in pilin assembly. PilD is particularly interesting, since it has been demonstrated as possessing peptidase activity (Nunn and Lory 1991), specific to the amino terminal sequence of the prepilin subunit, PilA. The cleavage site, and sequence requirements have been well characterised (e.g. Strom and Lory 1992). Similar pilin assembly clusters have also been identified in *Neisseria gonorrheae* (Dupuy *et al.*, 1992) and *Vibrio cholerae* (Kaufman *et al.*, 1991).

1.9.2 The search for Out protein functions

1.9.2.1 Localisation

An important clue in establishing functions for the Out proteins and their homologues would be their localisation in relation to the inner and outer membranes. Table 1.4 (from Reeves *et al.*, 1993) shows predicted protein sizes and

numbers of hydrophobic stretches of an adequate size to be transmembrane. Many of the Out proteins (e.g. G to N) have a single hydrophobic stretch, in many cases close to the amino-terminal, which could act as a membrane anchor. These hydrophobicity profiles are well conserved amongst the various systems studied. OutF appears to have three transmembrane segments, while OutO has eight. OutE is predicted to be cytoplasmic, since it has no significant hydrophobic stretch, and no feasible signal sequence. Attempts to localise many of the components have been misleading. PulG, H, I, J. K, L, M, N, and O have all been reported to cofractionate with the inner membrane (Pugsley and Reyss 1990; Reyss and Pugsley 1990), although many were also found in small quantities in the outer membrane fraction. It was suggested at the time that the root of the problem could be the formation of a complex spanning the two membranes, which may well be true, however, recent evidence suggests that it is due at least partly to experimental design. Pugsley and Dupuy (1992) report a modification of the T7g10p method used: induction was reduced to five minutes, to limit the extent of overexpression; rifampicin was not used, so that endogenous membrane proteins could be visualised, and used as controls for membrane fractionation; and spheroplasting and osmotic shock were used as opposed to sonication, which can cause fusion of membrane vesicles. The result was that PulG was shown to cofractionate significantly with the outer membrane. The problem with this method, though, is that PulG is much more highly abundant than the other Pul components, so that visualisation of these will be more

difficult. The key to providing a complete picture therefore seems to lie in the raising of antibodies, which, because of the difficulties in the purification of membrane proteins, presents problems of its own.

1.9.2.2 OutD homologues

OutD and its homologues are predicted to contain potential signal sequences and a high proportion of B sheet-forming sequences (Reeves *et al.*, 1993), as previously described in several outer membrane proteins, including *E. coli* porin (Kleffel *et al.*, 1985; Paul and Rosenbusch 1985). Localisation studies for PulD (d'Enfert *et al.*, 1989) confirm the prediction that this is therefore an outer membrane protein. However, the idea that it may form a polymeric pore is more difficult to envisage since, for example, PulD is approximately 71kDa, while pullulanase is 116kDa, which means that a high number of monomers would need to associate in order to form a pore of adequate size. In addition, such a pore would need to be gated in some way, to effect specific secretion.

1.9.2.3 OutE homologues

OutE, PulE, ExeE, XpsE, XcpR, and PilB all contain consensus motifs for Walker Boxes A and B (Walker et al., 1982), which are thought to form a nucleotide binding site. In addition, the Walker Box A motif is present in the more distantly related PilT, ComG-1 and VirB-11, ATPase activity having been demonstrated for the last of these (Christie *et al.*, 1991). This ATPase activity might energise secretion itself,

or provide the energy required for the assembly of the secretion apparatus. The latter is supported by the evidence (Possot *et al.*, 1992) that PulE must be made at the same time as the other components, and will not rescue secretion if added later.

1.9.2.4 OutO homologues

As shown in figure 1.8, OutO is homologous to PilD, a peptidase of *Pseudomonas aeruginosa* that is required for processing of the pilin precursor PilA, via cleavage of an N-terminal signal sequence. These are known as Me-Phe (typeIV) pili, since processing results in methylation of the phenylalanine at the amino-terminal of the mature protein. This is particularly interesting since the consensus target for Me-Phe processing is strongly conserved in OutG, H, I and J, and their homologues in all systems so far studied. Processing of at least one of these (usually the OutG homologues, since this is more abundant) has indeed been demonstrated for OutO of Ecc (Reeves *et al.*, in press); XcpA (Bally *et al.*, 1992) and PulO (Pugsley and Dupuy 1992). Southern hybridisation has identified a homologue in *Neisseria gonorrheae* pilin assembly, and the pilin precursor of this species can be processed in *E. coli* by PulO, ComC and XcpA (Dupuy *et al.*, 1992). In addition, alkaline phosphatase fusions to the prepilin have shown that considerable changes to the mature segment do not affect this processing event.

As to a role in secretion, several hypotheses have been proposed, though these lack experimental support. Various

workers (Possot *et al.*, 1992; Albano *et al.*, 1989) speculate that OutG-J homologues might assemble into a pilus-like structure, which could either be involved in guiding the secreted proteins, or in the formation of temporary contact zones between the two membranes, in order to facilitate secretion (Reeves *et al.*, 1993).

1.10 Evidence for a two-step mechanism

The original evidence for a two-step secretion mechanism for some of these extracellular proteins was indirect. Firstly, protein precursors possess a classical signal sequence at the amino terminal, as required in the Sec pathway. Secondly, expression of such proteins in *E. coli* results in the build up of a periplasmic form with the signal sequence cleaved (e.g. Barras *et al.*, 1984; Pugsley *et al.*, 1991a). And, thirdly, the Out$^-$ phenotype in Erwinia spp. results in periplasmic accumulation of Cel and Pel, with their signal sequences removed (e.g. Ji *et al.*, 1987). However, since these observations are based on Out$^-$ situations, an alternative explanation for the apparent two-step nature of secretion would be that the Out machinery also provides a means of inner membrane translocation, the Sec machinery merely providing a default pathway in its absence.

He *et al.*, (1991b) used pulse-chase radioactive labelling of PelE to show momentary localisation in the periplasm; and also showed that overexpression of PelE in either *Ech* or *E. coli* Out+ resulted in periplasmic accumulation. (This is also true for CelV of *Ecc*, but not for EGZ or *Ech* (Py *et*

al., 1991a)). In addition, a PhoA fusion indicated that the amino-terminal (including about 70% of PelE) was not sufficient to direct secretion; and that blocking of this hybrid in the periplasm did not interfere with the secretion of wildtype PelE, suggesting that it is free in the periplasm.

SecA, D, E, F, and Y, and GroEL have been shown to be necessary for pullulanase secretion by the analysis of temperature sensitive mutants (working in *E. coli* carrying the entire *pul* gene cluster; Pugsley *et al.*, 1991a). In a particularly elegant experiment (Pugsley *et al.*, 1991b) in *E. coli*, *pulA* was expressed under maltose control, while the other *pul* genes were expressed on a different plasmid under IPTG control, thus allowing uncoupling of PulA synthesis and secretion: growth in maltose results in accumulation of pullulanase in the periplasm; then when maltose is removed from the medium and IPTG added, secretion occurs. The fact that maltose is removed allows us to conclude that the pullulanase which is now secreted is that which had accumulated in the periplasm, indicating that export via the GEP results in a bona fide secretion intermediate. In the opposite experiment, in which the same strain was grown in IPTG, and then maltose was added while was IPTG removed, secretion was still efficient, indicating that the Pul secretion apparatus proteins are stable. This is important in the context of the work using Sec mutants (Pugsley *et al.*, 1991a) since it shows that these mutants do not exhibit a secretion-deficient phenotype simply by preventing the assembly of the Pul apparatus.

1.11 The problem of specific secretion

In order for Cel and Pel to be specifically secreted by the Out apparatus, there must be some kind of specific interaction between the secreted proteins and one or several of the Out proteins. This idea, however, presents a paradox in that there exists considerable sequence homology between the enzymes of any chosen class (e.g. Pel, Cel, or Peh) between species, and yet the enzymes of one species are not secreted by the other (e.g. Py *et al.*, 1991a). On the other hand, there is no obvious homology between the members of different classes of enzyme in a given species, yet they are secreted by the same Out apparatus. The obvious conclusion is that any motif required for recognition by the Out proteins cannot be in the form of an obvious linear sequence.

1.12 The nature of the secretion signal

By looking at work in other systems, a number of hypotheses could be formulated regarding the nature of the motif required for recognition by the Out apparatus. The first is that this is in the form of a strictly linear sequence, similar to the classical signal sequence, functioning independently of the nature of the "passenger" sequence which it directs. The second is that it consists of a discrete region of the protein, but is more dependent on conformational integrity, as seems to be the case in haemolysin secretion. The lack of sequence homology between Cels and Pels provides adequate evidence to disregard both of these ideas. The third idea is that the secretion signal

is a structural motif not recognisable in the primary sequence, which is by its very nature dependent to some extent on overall conformation. The fourth is that it is made up of several amino acids located at distant sites in the primary sequence, which rely on overall conformation of the molecule to bring them together to form the required motif. These are both feasible hypotheses, and will be discussed in more detail in the following pages.

1.13 Why work on CelV?

CelV of *Ecc* was chosen for this study primarily because it accounts for at least 95% of CMCase activity detected (we have preliminary evidence (S. Jones, pers. com.) for a second minor cellulase of very low activity, as seen in other strains of *Ech* and *Ecc* (Guisseppi *et al.*, 1991; Saarilahti *et al.*, 1990b)). This compares with a complex network of pectolytic enzymes, including (at least) 4 Pel isozymes. This would mean that in order to study secretion of one Pel, the genes encoding all of the others would have to be disrupted, in order to prevent them from masking the one under study. So, despite the fact that Pels are extremely important from the point of view of virulence, it makes more sense to study secretion by the Out apparatus using cellulase.

1.14 Characteristics of CelV

Appendix 1 shows the sequence of *celV* (Cooper and Salmond, 1993). The basic characteristics of CelV are illustrated in figure 1.10. It consists of 4 functional regions: an N-

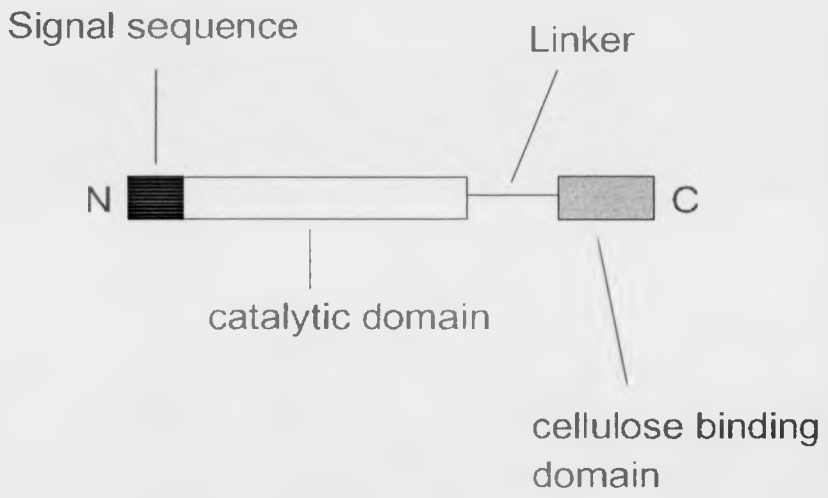


Figure 1.10. The domain organisation of CelV.

terminal signal sequence cleaved during passage across the inner membrane; a large catalytic domain at the N-terminal of the mature protein; and a C-terminal cellulose binding domain (CBD). The latter two functional domains are separated by a linker region rich in proline and threonine.

The catalytic domain shows a high level of homology to that of the endoglucanase EGZ of *Ech* (43% amino acid identity over a 214 amino acid stretch in this domain). Both cellulases are in the catalytic family A (Henrissat *et al* 1989), on the basis of hydrophobic cluster analysis, and a search for conserved Asp, Glu and His residues among members of this family (Henrissat *et al.*, 1989) has resulted in the identification of putative active site residues. Several of these, e.g. His98 and Glu133 of EGZ (His98 and Glu136 of CelV) have been confirmed by site directed mutagenesis (Py *et al.*, 1991b).

The CBD of CelV is identified as such by virtue of its homology to one of the domains in the cellulose binding region of the avicellase CelZ of *Clostridium stercorarium* (Jauris *et al.*, 1990). This enzyme has demonstrated binding properties to HBS cellulose (Serva, Heidelberg), to the extent that this was utilised during purification. CelV also shows a limited ability to bind HBS, but this proved to be highly dependent on the conditions used, and not highly specific (see chapter 4).

1.15 Previous secretion studies on cellulases

Very few studies have as yet focussed on the proteins

secreted by Out-type machinery, and results so far by no means provide us with a clear picture. In an extensive study of EGZ of *Ech*, Py *et al.*, (1993) constructed a series of terminal and internal deletions, using the domain structure of the protein as a framework, the aim being to try to narrow down the region containing the secretion motif(s). All of the deleted proteins exhibited catalytic or cellulose binding activity, depending on the domain deleted, and were shown by immunoblot to be stable and therefore not grossly misfolded, but none were secreted. In addition, in further constructs where the linker was deleted or lengthened, domain function and stability were again retained, but secretion was blocked. They concluded that there must be secretion information in both domains; and that the linker played a specific role, either directly, or in influencing the positioning of information in the two domains in relation to each other. None of the recombinant forms blocked the secretion of the wild type form, indicating that they are all deficient at the recognition stage, as opposed to translocation. This study also indicates a reliance on at least some degree of periplasmic folding, in that the integrity of domains is important, and that information is probably located at distant sites, brought together in a specific manner. Further evidence for periplasmic folding of secreted proteins will be discussed later.

Huang and Schell (1992), working on endoglucanase EGL of *Pseudomonas solanacearum*, have presented somewhat different

conclusions. They provide evidence for a "two-component leader sequence", the first 19 residues being cleaved by signal peptidase II and the remainder on crossing the outer membrane. However, they also show evidence for a reliance on the conformational integrity of the protein in order for trans-location across the outer membrane to be effected.

1.16 Evidence for dependence on periplasmic folding

Both of the examples above provide clues that integrity of the protein is important for secretion, and this suggests that at least some degree of folding occurs in the periplasm in order for a protein to be in a secretion compatible state. This theory is supported simply by the fact that stable periplasmic intermediates can be isolated (e.g. Poquet *et al.*, 1993). In addition, several studies have indicated that prevention of the formation of di-sulphide bridges in the protein to be secreted abolishes secretabiliy (Pugsley (1992), working on pullulanase secretion; Bortoli-German *et al.* (in press), working on EGZ of Ech; Drefus *et al.*, (1992), working on STb enterotoxin of *E. coli*, responsible for its own secretion).

Proteins responsible for catalysis of periplasmic formation of disulphide bridges have been identified in several systems. Bardwell *et al.*, (1991) have demonstrated that the disulphide oxidoreductase encoded by *dsbA* of *E. coli* is not essential to cell survival, but is important in the correct periplasmic folding of several proteins studied, *B*lactamase, alkaline phosphatase and OmpA. In DspA-defective mutants,

release into the periplasmic space is normal, but the proteins are susceptible to protease and only chase very slowly into stable oxidised forms. Kamitani *et al.*, (1992) report defective release into the periplasm and increased protease susceptibility of PhoA. In other systems, the role of DsbA homologues in folding has been linked to secretion competence. For example, Peek and Taylor (1992) reported that the mutation of the *dsbA* homologue of *Vibrio cholerae* interfered with the ability of secreted virulence factors, including cholera toxin, to assume secretion and functionally competent states. In addition, Pugsley (1992) has demonstrated that mutation of *dsbA* interferes with the secretion of Pullulanase, when *pulA* and the secretion genes are expressed in *E. coli*.

The reliance on folding also points to a role for periplasmic chaperone proteins, a role which could be fulfilled by one or more of the Out proteins. Kuehn *et al.*, (1991) have reported the characterisation of a periplasmic chaperone, PapD, required for pilus assembly in *E. coli*. It appears that in associating with 6 or more pilus subunits, it functions to partition newly translocated subunits, preventing non-productive aggregation that otherwise occurs, and instead forming assembly-competent complexes. Unlike cytoplasmic chaperones, PapD appears to effect its function by maintenance of PapG in a native-like conformation.

1.17 Introduction to this project

The aim of this project was to investigate the basis of

specific secretion of cellulase CelV by the Out apparatus. The starting point for the aims of this project was the investigation of secretion of EGZ secretion by F. Barras and coworkers. While this project was underway, Py *et al.* (1993), as already discussed, provided evidence, with the use of deletions, that both the CBD and catalytic domain possess information for secretion, and that presentation of this information was conformationally dependent. In addition, Bortoli-German *et al.* (in press) have demonstrated the importance of the periplasmic formation of a disulphide bond in the CBD in order for secretion to occur. Based on the high degree of homology in the N-terminal portions of EGZ and CelV, their similar domain organisation, and homologies between the Out machineries responsible for their secretion, it might be reasonable to assume that evidence about EGZ secretion can be directly applied to the secretion of CelV. However, there are several lines of evidence that suggest that this may not be the case. Firstly, the secretion of CelV can be overloaded by its expression on a high copy number plasmid, while EGZ is efficiently secreted under these conditions. Secondly, CelV possesses no cysteine residues to function in the way that those of EGZ appear to function. Thirdly, the Out machineries of the two species, although having considerable similarities, are, nevertheless significantly different, as already discussed.

The primary aim of the project was to investigate the nature of the information required for the secretion of CelV by taking an alternative approach: The isolation of point mutants deficient in secretion. Information gained from the

characterisation of such mutants could then hopefully be used to implicate specific amino acids as being involved in a recognition motif for secretion by Out. Such mutants could then be used in the isolation of intragenic suppressors, to expand the information about the secretion motif of CelV, and extragenic suppressors, to try to identify proteins which interact with CelV in order for secretion to occur.

The study of secretion first required the establishment of a system amenable to study. This required the isolation of polyclonal antibodies against CelV, and the establishment of a plasmid-based expression system which does not overload the secretion apparatus, which in turn required a well characterised cellulase deficient mutant for use as the background for studies.

The study of CelV secretion initially required collaboration with several laboratory members (Reeves *et al.*, 1993) to complete the sequence of the cluster of genes encoding the Out apparatus, both to complete the picture of the system under study, and to provide information which might be necessary for the characterisation of extragenic suppressors. It was also important to verify that the findings of the deletion studies of Py *et al* (1993)., using EGZ also held true for CelV, by the isolation of several deletions of a similar nature. Lastly, a parallel study was the investigation of the role that CelV plays in the virulence of *Ecc*.

CHAPTER 2

MATERIALS AND METHODS

2.1. MEDIA AND GROWTH CONDITIONS

2.1.1 Media

Growth media and solutions were prepared in Elga purified water, and are listed in table 2.1. Where necessary, media and solutions were sterilised by autoclaving at 121°C for 20 minutes. LB and NB were solidified by the addition of 1.6% (w/v) Bacto agar. For *Erwinia* phage work, 0.5% (w/v) agar LB lawns were used. For *E. coli* phage work, DDA and soft DDA contained 0.9% and 0.3% (w/v) Bacto agar. All phage media were supplemented with magnesium sulphate to a final concentration of 0.01M. Antibiotics were prepared as 100 X stock solutions, and used as described in table 2.3.

2.1.2 Growth conditions

Erwinia spp. and *E. coli* were grown on LB agar containing appropriate antibiotics, at 30°C and 37°C, respectively. Liquid cultures were grown in Universal (25ml) screw cap tubes containing 5ml LB, or in conical flasks if the volume was larger. All were shaken in an orbital shaker (250rpm) or in a vertically rolling carousel.

2.1.3 Storage conditions

All *E. coli* and Ecc strains were maintained on LB plates at 4°C for up to 3 months. Long term storage was by freezing cultures with an equal volume of 2 x freezing medium (table 2.1) at -70°C.

Medium/solution	Constituents per litre[1]
NBA	13g Oxoid nutrient brith 16g Bacto agar
LB	10g Bacto tryptone 5g bacto yeast extract 5g NaCl (pH7.2)
LBA	16g Bacto agar for plates 5g Bacto agar for top lawns
LBSE	10g Bactotryptone 5g Bacto yeast extract 58.5g NaCl 0.37g EDTA (pH7.2)
DDA	20g Bacto tryptone 8g NaCl (10ml 1M $MgSO_4$)
DDA agar	9g Bacto agar for plates 3g Bacto agar for top lawns
SOC	20g Bacto tryptone 5g yeast extract (10ml 1M NaCL) (2.5ml 1M KCl) (10ml 1M $MgSO_4$, 1M $MgCL_2$) (20ml 1M glucose)
TB Mg	10g Bacto tryptone 5g NaCl (10ml 1M $MgSO_4$)

Table 2.1 General solutions and media.

[1] Constituents in brackets are made up separately and added after autoclaving.

Medium/solution	Constituents per litre[1]
Phage buffer	10mM Tris(hydroxymethyl)aminoethane 10mM $MgSO_4$ 0.01% (w/v) gelatin (pH7.4)
Minimal medium	(20ml 50 x phosphate) (10ml 10% (w/v) $(NH_4)_2SO_4$) (10ml 1% (w/v) $MgSO_4.7H_2O$)
50 x phosphate	350g K_2HPO_4 100g KH_2PO_4 (pH6.9 - 7.1)
Freezing medium(2 x)	12.6g K_2HPO_4 0.9g sodium citrate 0.18g $MgSO_4.7H_2O$) 1.8g $(NH_4)_2SO_4$ 3.6g KH_2PO_4 88g glycerol
2YT	16g Bacto tryptone 10g yeast extract 5g NaCl 15g Bacto agar for plates
TBE (10 x)	108g Tris(hydroxymethyl)aminoethane 55g Boric acid 9.3g EDTA
Ligation buffer (1 x)	66 mM Tris.HCl pH7.6 6.6 mM $MgCl_2$ 10mM DTT 0.4 mM ATP

Table 2.1, continued.

[1] Constituents in brackets are made up separately and added after autoclaving.

Medium	Constituents per litre[1]
Pectinase plates	16g Bacto agar (5ml 20% (w/v) yeast extract) (10ml 10% (w/v) $(NH_4)_2SO_4$) (1ml 1M $MgSO_4$) (10ml 50% (w/v) glycérol (250ml 2% (w/v) polygalacturonic acid) (200ml pel phosphate buffer)
Pel minimal broth	(5ml 20% (w/v) yeast extract) (10ml 10% (w/v) $(NH_4)_2SO_4$) (1ml $MgSO_4$) (9ml 50% (w/v) glycerol) (125ml 2% (w/v) polygalacturonic acid) (100ml pel phosphate buffer)
Pel phosphate buffer	15g Na_2HPO_4 0.7g $NaH_2PO_4.H_2O$ (pH8)
Cellulase plates	10g carboxymethyl cellulose (Sigma) 16g Bacto agar (25ml 20% (w/v) bacto yeast extract) (4ml 50% (w/v) glycerol) (20ml 50 x phosphate) (10ml 10% (w/v) $(NH_4)_2SO_4$) (10ml 1% (w/v) $MgSO_4$)
Protease plates	13g Oxoid nutrient broth 30g Oxoid gelatin 16g Bacto agar

Table 2.2 Enzyme detection media.

[1] Constituents in brackets are made up separately and added following autoclaving.

Antibiotic	Final concentration[1]	Storage
Ampicillin (Na)	50ug/ml	4°C
Chloramphenicol	50ug/ml	-20°C
Kanamycin sulphate	50ug/ml	4°C
Spectinomycin	50ug/ml	4°C
Tetracycline	10ug/ml	-20°C

Table 2.3 Antibiotics.

[1] Tetracycline and chloramphenicol are dissolved in 50% (v/v) ethanol, while others are dissolved in water. All are filter sterilised.

2.2 ACTIVITY ASSAYS

2.2.1 Cellulase detection plates

The method was modified from Gilkes *et al.* (1984) by J. Hinton (pers. com.). The constituents are shown in table 2.2. The carboxymethyl cellulose (medium viscosity, Sigma), agar and water were shaken together vigorously, autoclaved, and cooled to about 55°C before addition of the other components, then poured into petri dishes as usual.

The detection plates were developed by flooding with 0.2% (w/v) Congo Red (Sigma) for 20 minutes, bleaching for 15 minutes with 1M NaCl and then fixing with 1M HCl for 5 minutes. Cellulase activity was identified by the appearance of a pink translucent halo against a red (if not fixed with HCl) or dark blue (if fixed) background.

2.2.2 Protease detection plates

The method was that of Thurn and Chatterjee (1985), using the constituents given in table 2.2. The detection plates were developed by flooding with 4M ammonium sulphate, a clear halo on a white opaque background indicating the presence of protease activity.

2.2.3 Pectate lyase detection plates

The method used was as described by Andro *et al.* (1984), using the constituents given in table 2.2. The detection plates were developed by flooding with 7.5% copper acetate

(Sigma) for up to 30 minutes. the presence of a pale double halo on a blue background indicating the presence of pectate lyase activity.

2.2.4 Cell fractionation

Strains were grown in LB to stationary phase (optical density of A_{600} = 1.2 - 1.4) and centrifuged (4,500rpm in a Labor 50M, Wifug) and the supernatant removed. The resulting pellet was washed once with LB, then used to prepare either a cytoplasmic + periplasmic fraction (by sonicating, as below) or separate cytoplasmic and periplasmic fractions as described below.

Spheroplasting was carried out as described by Sambrook *et al* (1991). The cell pellet was resuspened in one tenth of the original volume of 30mM Tris HCl pH8, 1mM EDTA pH8, 20% sucrose , 1mg/ml lysozyme (Sigma), and incubated on ice for 10 minutes before centrifugation, the resulting supernatant representing the periplasmic fraction. The cell pellet was the washed in LB, and resuspended in the original volume of LB. It was sonicated for 3 x 30 seconds at an amplitude of 6 with a 0.7 inch probe at 4°C, with 30 seconds rest between each sonication. Cell debris was removed by centrifugation (in a Labor 50M (Wifug)) at 4,500rpm, 10 minutes. Fractions were stored at -20°C.

2.2.5 Cellulase assays of cell fractions

Two methods were chosen, which were mutually complementary.

The Ostazin Brilliant Red-Cellulose method is very sensitive but occasionally unreliable, while the well assay method is very reliable but less sensitive. In both cases a standard curve was constructed using serial dilutions of SCR1193 whole culture sonicate, and results were expressed as percentage SCR1193 total activity.

Ostazin Brilliant Red-Cellulose assay

This method is based on that of Biely *et al.* (1985), adapted by F. Ellard (pers. com.). The substrate OBR-cellulose (Sigma) is broken down by cellulase to release a soluble red pigment, which can be measured at 550nm following centrifugation to pellet the cellulose. The reaction mix was as follows.

280µl OBR-cellulose, 1.025% (w/v)
70µl cell fractionation sample
7µl 50 x phosphate buffer

Samples were incubated at 30°C for 80 minutes, then the reaction stopped with 3 volumes of 2:1 ethanol:acetone. Following incubation for 5-30 minutes at room temperature, samples were centrifuged at 12,000rpm in a microfuge for 10 minutes at 4°C. The supernatants were removed and measured spectro-photometrically at 550nm against a water blank.

Cellulase and pectate lyase well assays

Pectate lyase and cellulase were assayed by making wells 6mm in diameter in pectate lyase or cellulase detection plates,

using the wide end of a sterile pasteur pipette. Wells were inoculated with 50ul of fractionation sample, and incubated at 37°C for 48 hours, before being developed as usual.

2.2.6 Pectate lyase spectrophotometric assay

As for cellulase, this assay was supported by the data from pectate lyase well assays. The spectrophotometric method was obtained from J. Hinton (pers. com.), based on that of Chatterjee *et al.* (1985). It follows the breakdown of polygalacturonate (PGA) to digalacturonate, which results in an increase in absorbance at 235nm. The following were placed in a quartz cuvette.

295µl reaction mix (preheated to 37°C)
7.5µl fractionation sample

Cuvette contents were mixed by inversion and inserted into the temperature-controlled cuvette holder of a Philips PU 8720 scanning spectrophotometer. The absorbance was followed at 235nm, and the initial reaction rate obtained from the gradient of the slope during the early stages of the reaction. Serial dilutions of SCR1193 whole culture sonicate were used to construct a standard curve, allowing results to be expressed as percentage SCR1193 total activity.

Reaction mix: 3.45ml reaction buffer
3.20ml 0.57% polygalacturonate
1.13ml water

Reaction buffer: 76.8ml water
23.0ml Tris.Hcl, 1M, pH8.5
78.0µl calcium chloride

2.2.7 *B* lactamase assay

This assay was carried out as described by O'Callaghan *et al* (1972). The reaction mixes were as follows.

Reaction mix: 100µl sonicate, supernatant, or 1:10 dilution of periplasmic fraction
700µl phosphate buffer, 0.1M, pH7

20µl of the chromogenic substrate Nitrocephin (4mg/ml in DMSO) was added to each reaction, then the rate of change of absorbance followed using a Philips PU 8720 scanning spectrophotometer at 500nm.

2.2.8 *B* galactosidase assay

This assay was based on Miller (1972). The reaction mixes were as follows.

Reaction mix: 450µl Z buffer
50µl sonicate

or

250µl Z buffer
250µl supernatant/periplasm

Z buffer (per litre): 8.52g Na_2HPO_4
6.24g $NaH_2PO_4.2H_2O$
0.75g KCl
0.25g $MgSO_4.7H_2O$
2.7ml mercaptoethanol (pH7)

100ul of fresh ONPG (Sigma, 4mg/ml) prepared with Z buffer, was added to each reaction. Reaction mixtures were then incubated at 37°C until they turned slightly yellow. The reactions were stopped using 250µl Na_2CO_3, and the rate of change of absorbance calculated using a Philips PU 8720 scanning spectrophotometer at 420nm.

2.3 DNA TECHNIQUES

2.3.1 Small-scale preparation of plasmid DNA (miniprep)

Small-scale plasmid preparations were carried out by alkaline lysis using a method based on that of Maniatis *et al.* (1982) (R. Barallon, pers. com.). Between 1.5 and 3ml (depending on the copy number of the vector to be isolated) of *E. coli* or *Erwinia* spp. cell culture , grown overnight, was pelleted in 1.5ml microfuge tubes at 12,000rpm for 2 minutes. The supernatant was discarded and the pellet resuspended in 100ul of lysis solution (25mM Tris.HCl, pH8; 50mM glucose; 10mM EDTA, 2mg/ml lysozyme) and incubated for 5 minutes on ice. 200µl of 0.2M NaOH/1% SDS was added and the solution mixed by inversion. 150µl of 5M sodium acetate solution (adjusted with glacial acetic acid to pH 4.8, giving a solution which is 3M sodium, 5M acetate) was added and the solution vortexed briefly then incubated on ice for 5 minutes before centrifugation in a microfuge at 12,000rpm for 10 minutes. The resulting supernatant was transferred to a clean microfuge tube and an equal volume of phenol/ chloroform added and the tube vortexed. Following 2 minutes centrifugation, the top, aqueous layer was transferred to a

clean tube. Phenol/chloroform treatment was repeated as necessary, up to 3 times. To the aqueous solution, one tenth volume of sodium acetate (as above) was added, followed by 3 volumes of ice cold ethanol. DNA was precipitated by incubation of the tube at -70°C for 30 minutes, and then centrifuged at 12,000rpm for 10 minutes. The supernatant was carefully removed, and the pellet washed with 70% ethanol. Following removal of this, the pellet was dried in a 55°C oven for 5 minutes (or under vacuum for 15 minutes) and then resuspended in 100ul of TE, pH7.5, containing 20µl/ml RNAse I and stored at 4°C or 20°C.

2.3.2 "Magic Miniprep"

A Magic Miniprep kit, supplied by Promega was used for small-scale preparation of plasmid DNA for low copy number vectors, such as pDEE339 and derivatives, and cosmids. In addition, owing to the high level of purity of product and the inexpensiveness of the kit, it was also used to prepare pUC19 derivatives for sequencing, instead of the method below, when only a small amount of DNA was needed. The method used was as described by the manufacturer.

2.3.3 Large-scale plasmid preparations (maxipreps)

Large-scale preparation of plasmid DNA was carried out using Qiagen "plasmid" Midi kits (Diagen). This method was preferred to conventional caesium chloride gradients for several reasons: preparation takes 3-4 hours, compared with 2 days; and the resulting DNA is of a high quality, suitable

for sequencing. In addition, for the low copy number pDEE339 derivatives a greater and more reliable yield is obtained.

2.3.4 Restriction endonuclease digestion

The restriction endonuclease digestion of DNA was carried out according to the manufacturers' instructions (BRL; Pharmacia; Boehringer Mannheim). All digests were performed at the temperature recommended by the manufacturers for 1 or 2 hours. When digestion with 2 or more enzymes was required, double digests were performed whenever compatible restriction buffers allowed.

2.3.5 Agarose gel electrophoresis of DNA

DNA samples were prepared for electrophoresis by addition of quarter volume of loading buffer (0.25% w/v bromophenol blue; 40% w/v sucrose). Unless otherwise stated, samples were electrophoresed in a 0.75% agarose gel in TBE buffer (1.08% tris (hydroxymethyl) aminoethane; 0.55% boric acid; 0.09% EDTA) containing ethidium bromide (0.25ug/ml final concentration). The gel was prepared by boiling the constituents, cooling to 55°C, adding ethidium bromide, and pouring into a gel mould (Biorad Subcells or Minisubcells). Gels were electrophoresed at 90V until good separation was achieved. The molecular weight standards were 1kb Ladder (BRL). Gels were visualised under long wave UV, and photographed under short wave UV, using Polaroid 665 positive/negative film.

2.3.6 Isolation of DNA from agarose gels

Two alternative methods were used, Geneclean, as described in section 2.3.7, or elution (R. Barraion, pers. com.). A slit was cut just ahead of the required band. Whatman 3mm paper and a single piece of dialysis membrane (section2.8.2) were cut to the width of the slot and slightly deeper than the gel, and soaked in TBE for 5 minutes. They were inserted into the slit, the 3MM paper nearest the DNA and electrophoresis was continued until the required band disappeared into the paper. The paper and membrane were placed in a 400µl Ependorf tube inside a 1.5ml Ependorf tube. These were centrifuged for 15 seconds at 12,000rpm, and the eluant collected. 2 aliquots of 100µl of elution buffer were then added to the small tube, and after each addition the tubes were centrifuged and the eluant collected as before. The pooled eluant was extracted with phenol, phenol-chloroform and chloroform, ethanol precipitated and ethanol washed, by standard methods (Maniatis *et al*, 1982) and resuspended in TE, pH7.6.

Elution buffer: 0.2M NaCl
50mM Tris.HCl, pH7.6
1mM EDTA
0.1% SDS

2.3.7 Geneclean

A Geneclean II kit (BIO-101) was used according to supplier's instruction both for the recovery of DNA from agarose gels, and purification of DNA between reactions

(e.g. following restriction digest or end repair, and before ligation).

2.3.8 End repair

"Sticky ends" were repaired using Klenow fragment, or blunted by the removal of nucleotides using either *E. coli* DNA polymerase I or Klenow fragment, depending on the overhang to be removed, all as described by Ausubel *et al.*, (1987).

2.3.9 Ligation

A fragment to vector ratio of approximately 4:1 was used. The reaction mix contained one tenth volume T4 DNA ligase (BRL), and one tenth volume 10 x ligation buffer (table 2.1). Ligations were carried out overnight at 15°C for blunt ends, or for upwards of 4 hours at room temperature for sticky ends.

2.4 DNA SEQUENCING

2.4.1 M13 dideoxynucleotide termination sequencing using randomly generated clones

Random DNA sequencing was performed using the protocol of Bankier *et al* (1986). The target DNA was first broken down into random fragments which were then cloned into M13 for sequencing of single stranded M13 clones using Universal primer and a Sequenase kit V2.0 (United States Biochemical Corporation).

2.4.2 Circularisation of target DNA

The aim of this procedure was to ensure that the fragments produced by sonication (section 2.4.3) were fully representative of the target DNA. The target fragment of DNA (see chapter 3 for details of these) was self ligated as described in section 2.3.9.

2.4.3 Sonication

The ligation mix resulting from section 2.4.2 was sonicated using a cup horn sonicator, at output 10 on a continuous cycle. The sample was sonicated for 3 x 60 seconds, with 30 second cooling intervals, on ice.

2.4.4 End repair of sonicated DNA

This was effected by incubating the sonicated DNA with DNA polymerase (Klenow fragment) as described in section 2.3.8.

2.4.5 Size selection

The sonicated and end repaired sample was electophoresed on a 1% agarose gel (section 2.3.5), using lambda DNA cut with PstI as molecular weight standards. The region of the gel corresponding to molecular weights of between 300 and 600bp was removed and the DNA recovered using an Electroelutor, (Biorad) eluting at 100V for 20 minutes until DNA could no longer be visualised under UV in the gel slice.

2.4.6 Plasmid sequencing

Plasmid sequencing of cloned double stranded DNA fragments was performed using the following method (V. Mullholland, pers. com), based on Mierendorf and Pfeffer (1987). Plasmid DNA was prepared using either Qiagen maxipreps (section 2.3.3) or Magic Minipreps (section 2.3.2).

2.4.7 Preparation of single stranded template DNA

To 3ug of plasmid DNA, a quarter volume 1M NaOH, 1mM EDTA was added, and the reaction incubated at 37°C for 30 minutes to denature the plasmid DNA. One tenth volume of 3M sodium acetate (pH4.8) was added to neutralise the NaOH, and three volumes of ethanol added, followed by precipitation at -70°C for 15 minutes. Following centrifugation at 12,000rpm in a microfuge, the supernatant was carefully removed and discarded, and the pellet washed once in 70% (v/v) ethanol before drying under vacuum. The pellet was then resuspended in 7µl water.

2.4.8 Sequencing reactions using "Sequenase"

Reactions were performed using a Sequenase V2.0 kit (United States Biochemical Corp.). Annealing reactions consisted of 7ul single stranded template, 1ul reaction buffer (from the kit) and 2ul primer. They were incubated at 37°C for 15-30 minutes. The remainder of the sequencing protocol was carried out as described by the manufacturers, using microtitre dishes to enable up to 12 reactions to be performed simultaneously. Completed reactions were stored at

-20°C until required, and heated to 80°C prior to loading onto a sequencing gel.

Primers used for this procedure were designed specifically to allow the sequencing of the entire *cellA* gene, and are listed in table 2.4.

2.4.9 Sequencing gels

1-5% gradient gels were poured using 50 x 20cm plates which had been thoroughly cleaned using dishwashing detergent, ethanol and acetone. The back ("eared") plate was siliconised using Replicote (BDH), allowed to dry, and then cleaned again with ethanol. Plates were assembled as described by the manufacturer (Biorad), using 0.4mm spacers.

Gradient gels were poured as follows. Solutions 1 and 2 were prepared separately, and ammonium persulphate (APS) and TEMED added as described to initiate polymerisation.

Solution 1: 7ml 5 x acrylamide
14µl APS, 25% (w/v)
14µl TEMED

Solution 2: 50ml 1 x acrylamide
100µl APS, 25% (w/v)
100µl TEMED

A 50ml syringe was used to withdraw 40ml of solution 2. A 10ml pipette was used to withdraw 5ml of the remaining solution 2, followed by 5ml of solution 1. An air bubble

Primer number	Position	Sequence
RU3	605-621	GGTCAGCTGTCCATCGA
RU7	1138-1154	TCAGCTGCCCGATCCGA
RU8	1154-1138	TCGGATCGGGCAGCTGA
RU9	1524-1540	AACCGACCAACCCGGGT
RU10	1540-1524	ACCCGGGTTGGTCGGTT
RU11	2059-2042	GAATTCACGCCCGCACG
DE1	704-688	AGTCACCAAACCACTGC
DE2	901-885	TGGGATCGTTATCCGAC
EXT1	1217-1201	CAATGCGGTCGCGCAGG
NU1	1773-1757	GCTGGCGTCCCCGTGCT
T3	pBluescript	ATTAACCCTCACTAAAG
T7	pBluescript	AATACGACTCACTATAG

Figure 2.4 Oligonucleotide primers.

The table shows, from left to right, the number of the primer; the position in the sequence of *celV* to which it anneals; and the 5' to 3' nucleotide sequence. Primers T3 and T7 are specific to the T3 and T7 promoters of pBluescript SK- (see figure 7.1), and were obtained from Stratagene.

was introduced to slightly mix the two solutions, then the contents of the pipette were poured in a slow, continuous stream down the side of the assembled gel plates. The contents of the syringe were then poured in a slow, continuous stream down the centre of the plates, holding the plates at an angle of 45°. When full, a shark's tooth well-forming comb was clamped in position at the top, and the gel left for 30-60 minutes to polymerise.

Before loading, the comb was removed, and the well rinsed with 1 x TBE, then the comb was replaced with the teeth downwards, to form wells, and each well was washed with 1 x TBE using a syringe and needle. The upper and lower buffer reservoirs of the electrophoresis apparatus (Biorad) were filled with 1 x TBE, and 2.5µl samples of sequencing reactions loaded into the wells using a Gilson. The gel was run at 38W for approximately 3 hours, until the leading dye front had reached the bottom of the gel. Gels were then fixed in 10% (v/v) acetic acid for 10 minutes, and dried under vacuum at 80°C for 20-30 minutes, using a gel drier (Biorad). Gels were then subjected to autoradiography for 18 hours or more, at room temperature.

Gel solutions

40% acrylamide stock solution (100ml): 38g acrylamide
2g bis-acrylamide

The solution was de-ionised by stirring over 2g amberlite resin (BDH) for 30 minutes, then filtered and stored at 4°C

in the dark.

1 x acrylamide gel mix (1l): 430g urea
100ml 10 x TBE
150ml 40% acrylamide stock

5 x acrylamide solution (1l): 430g urea
500ml 10 x TBE
150ml 40% acrylamide stock
30mg bromophenol blue

All acrylamide solutions were filtered, and stored at 4°C in the dark for up to 3 weeks.

2.5 TRANSFORMATION

2.5.1 Transformation of *E. coli*

The method used was based on that of Chung and Miller (1988) (I. Bortoli-German, pers. com). The strain to be used was grown in LB to A600 = 0.3-0.4, then pelleted and resuspended in one tenth volume of ice cold TSB, and incubated for 30 minutes on ice. Following the addition of 2-10µl DNA to 100µl of cells, the mixture was further incubated on ice for 30 minutes. Following the addition of 1ml of TSB, cells were allowed to express by incubating for 30-60 minutes at 37°C, shaking, then pelleted, resuspended in 100µl LB, and spread onto LB plates containing appropriate antibiotics. The plates were incubated overnight at 37°C.

TSB buffer: 10% Polyethylene glycol, Mr 6000 or 3500
5% Dimethyl sulphoxide
20mM Magnesium sulphate
20mM Magnesium chloride (pH 6.5)

2.5.2 Transformation of *Erwinia* spp.

Plasmid DNA was introduced into all species by electroporation (Solioz and Bienz 1990). Electroporation-competent cells were prepared by growing to A600 = 0.5-0.6, washing 3 times with sterile distilled water, once with 20% glycerol (halving the volume with each wash) and then resuspending in a volume of 20% glycerol approximately equal to the volume of cells. These were used immediately or stored at -70°C until required. Electroporations were performed using 1ul of DNA to 50ul of cells, using a Biorad Pulse Controller as described by the manufacturers' instructions. The conditions used were 2.5KV, 200ohms 25μFD. Following electroporation, the cells were immediately resuspended in 1ml SOC or SOB, and allowed to express by incubation at 37°C for 30-60 minutes. After pelleting, resuspending in 100μl LB, and spreading onto an LB plate containing appropriate antibiotics, the plates were incubated overnight at 30°C.

2.6 USE OF PHAGE LAMBDA

2.6.1 Preparation of a high titre lysate

Lambda phage lysates were propagated on the *E. coli* suppressing strain LE392. Two phage plaques were resuspended in 1ml of phage buffer (section 2.1) and various volumes

(between 1 and 100µl) added to 200µl aliquots of an overnight culture of LE392 grown in LB, 10mM magnesium sulphate. Following the addition of 3ml of soft DDA agar (table 2.1), the mixture was used to overlay a fresh, wet DDA plate (table 2.1) and incubated at 37°C for 16 hours. The plate showing confluent lysis was then harvested.

The top agar was removed using a glass spreader, the plate was washed with 3ml of phage buffer, and this was pooled with the agar. Following the addition of 0.5ml chlororform, the mixture was vortexed for 15 minutes to produce a soft slurry. Centrifugation of this (at 4,500rpm, 4°C in a Labor 5M (Wifug)) for 10 minutes resulted in pelleted cell debris and a clear supernatant, which was decanted. A few drops of chloroform were added, and the lysate stored at 4°C. The expected yield was approximately 10^{10} pfp/ml.

Lysates were titred by spotting serial dilutions onto freshly poured lawns of LE392 on DDA plates, and incubating at 37°C for 16 hours.

2.6.2 Transduction

When transducing Ecc, this requires the presence of the *E. coli lamB* gene. Strains were therefore first electroporated (section 2.5.2) with pHCP2, a pBR322 derivative containing *lamB* (Salmond *et al.*, 1986).

A fresh overnight culture of the relevant strain was diluted 1:20 in TBMg containing the required antibiotics and grown,

with shaking, at 30°C to A550 = 0.8. Cells were pelleted (4,500rpm, 5 minutes in a Wifug Labor 5M) and resuspended in 1ml of TBMg. Following the removal of 100ul as a control sample for spontaneous antibiotic resistance, 10^9 pfu of lambda was added and the mixture incubated at 30°C for 1-2 hours. 100µl aliquots were then spread onto plates containing the appropiate antibiotics and incubated at 30°C for 36 hours.

2.7 USE OF ERWINIAPHAGE

2.7.1 High titre lysates

An overnight culture was grown (in 5ml LB, 10mM $MgSO_4$) of an appropriate strain (phiKP stock was propagated on wild type SCRI193 or HC131). 10µl samples of serial dilutions of stock lysate were added to 200µl aliquots of cells, and incubated at ambient temperature for 10 minutes. Following addition of 4ml of soft top agar, these were poured onto fresh wet plates of LB agar, and incubated for 16 hours at 28°C. The plates exhibiting confluent lysis were then harvested.

The top agar layer was removed using a sterile glass spreader, and the plate washed with 2ml of phage buffer, which was then pooled with the top agar. Following the addition of 0.5ml chloroform the mixture was vortexed for 15 minutes then centrifuged for 10 minutes, 4,500rpm in a Labor 50M (Wifug) at 4°C and the supernatant decanted. This was stored at 4°C with a few drops of chloroform.

Lysates were titred by spotting serial dilutions onto fresh

lawns of SCR1193 and incubating at 26°C for 16 hours. The expected yield was 10^{10} to 10^{11} pfu/ml.

2.7.2 Transduction

The appropriate strain was grown overnight (in 5ml LB, 10mM magnesium sulphate), then inoculated with 100ul of a phiKP lysate and incubated at 26°C for 30 minutes. Cells were pelleted and washed in 10ml phage buffer, then resuspended in 10ml LB and expressed for 30-60 minutes at 30°C, shaking. Cells were pelleted and resuspended in 0.5ml LB, then spread onto plates containing appropriate antibiotics and incubated at 30°C for 48 hours.

2.8 HYDROXYLAMINE MUTAGENESIS

2.8.1 Mutagenesis of plasmid DNA

The method used was based on that of Oxender and Gibson (1991) (I. Bortoli-German, pers. com.). Plasmid DNA was prepared using one of the "miniprep" procedures described in section 2.3.1 then exposed to hydroxylamine in the following reaction conditions.

Reaction: 4 volumes hydroxylamine solution
1 volume DNA

Hydroxylamine solution: 350mg hydroxylamine
560µl NaOH, 4M
4.4ml water (pH 6)

The reaction was incubated at 37°C for a range of exposure times around 36 hours, then placed in dialysis tubing and dialysed against 10mM Tris. HCl pH7.4, 1mM EDTA, for 24 hours at 4°C, changing the solution about 4 times. The resulting DNA was then removed from the dialysis tubing and "Genecleaned" (section 2.3.7).

2.8.2 Preparation of dialysis tubing

(B. Fy, pers. com.). Dialysis tubing of a suitable width was cut into convenient lengths, and boiled in a large volume of 2% sodium hydrogen carbonate, 1mM EDTA for 10 minutes. It was then rinsed in distilled water and then boiled in distilled water for 10 minutes. On cooling, it was stored in the same water at 4°C until use. Before use, each piece was rinsed inside and out with distilled water.

2.8.3 Mutagenesis of high titre lysate

The protocol used (Housby 1993) was adapted for Ecc from Hong and Ames (1971). Following preparation of a high titre phiKP lysate, the following reaction was set up.

Reaction mix: 1ml high titre lysate
2ml phosphate-EDTA buffer
3ml sterile distilled water
4ml hydroxylamine solution

Phosphate-EDTA buffer: 6.0ml K_2HPO_4
43.9ml KH_2PO_4 (pH 6)

To this, an equal volume of 10mM EDTA was added, before autoclaving as normal.

Hydroxylamine solution: 560µl 4M NaOH
0.35g hydroxylamine
up to 5ml with water

Using the information gained from the construction of a killing curve (figure 9.7), the lysate was exposed to the mutagenic reaction mix for 22 hours by incubation at 37°C without shaking. Mutagenised phage were then precipitated by centrifugation at 17,000rpm for 2 hours in a Beckman Hi-spin, using a JA17 rotor. The supernatant was carefully poured off, and the tube drained by standing inverted on tissue for 5 or 10 minutes. The phage pellet was resuspended by adding 0.5ml phage buffer and incubating overnight at 4°C, then vortexing thoroughly.

2.9 GENERAL PROTEIN PROCEDURES

2.9.1 Protein concentration assay

The protein concentration of samples was calculated using the BioRad Protein Assay, as described by the manufacturers using a selection of known concentrations of bovine serum albumen to construct a standard curve.

2.9.2 HBS binding

The method used was based on that of Py *et al.* (1991b), the main change being that the last wash was with water as

opposed to urea, based on the observation (Cooper 1992) that CelV could be eluted from HBS cellulose using water.

1.5ml sonicated cell culture, grown in LB, was added to 24mg HBS cellulose (Serva, Heidleberg) and shaken gently for one hour at room temperature. This was centrifuged in a microfuge and the pellet resuspended in the same volume of 100mM Tris.Cl pH7.5; then centrifuged and the pellet resuspended in the same volume of 500mM NaCl. Following centrifugation, the pellet was resuspended in the same volume of water, and shaken for 10 minutes at ambient temperature, before centifugation. At each stage, the supernatant was retained for analysis.

2.9.3 Concentration of samples using Amicon Centriprep

Periplasmic samples were concentrated with the use of Amicon Centriprep 30 filters, as described by the manufacturers (Amicon Ltd).

2.9.4 T7 expression

CelV (or a mutant derivative) was overexpressed using the T7g10p system of Tabor and Richardson (1985). *celV* was cloned into pT7-6 (figure 4.1), and the resulting plasmid (pT7V-1 or a derivative) introduced into *E. coli* K38 already carrying pGP1-2 (figure 4.2). As the figure shows, in pT76, the gene to be overexpressd is cloned under the control of the T7 promoter; while on plasmid pGP1-2, T7 polymerase is under the control of a temperature sensitive promoter, P_L. Therefore, temperature induction of this promoter results in

synthesis of T7 polymerase, and thus overproduction of the gene in question.

K38 + pGP1-2 + pT7V-1 was grown in 2% yeast extract, 0.5% NaCl, 0.2% glycerol, 50mM pottassium phosphate pH7.2, 50ug/ml ampicillin, 50ug/ml kanamycin at 30°C to A_{595}=1.5. The culture was then induced at 42°C for 30 minutes and then expressed at 30°C for up to 150 minutes berore harvesting. All incubations were in an orbital shaking water bath, at 200rpm.

2.10 LARGE SCALE POLYACRYLAMIDE GEL ELECTROPHORESIS

2.10.1 Large scale gels

Large scale electrophoresis or proteins was carried out on 10% homogeneous gels as described by Silhavy *et al.* (1984). Samples were diluted 1:1 in loading buffer; heated 5 minutes in a boiling water bath; and electrophoresed using a Biorad Protean II apparatus. Gels were Coomassie stained ror 30 minutes and destained as necessary (Silhavy *et al.* 1984).

2.10.2 CMCase activity overlay

PAGE was carried out as described, the gel was then rinsed in distilled water, washed over two hours in several changes of 2.5% Triton, 10mM Tris HCl pH8 to remove SDS, and rinsed again in water. A CMCase activity overlay (Cooper 1992) was prepared by casting a gel or 0.5% carboxymethyl cellulose (Sigma), 0.1% agarose in 1.5 X phosphate buffer (see table 2.1) between glass plates lined on one side with Gelbond

(Hybaid). The gel was used to overlay the Triton-washed gel for one hour at 42°C, then stained for 30 minutes in 0.2% Congo Red (Sigma) and destained as necessary in 1M NaCl, before fixing with 1M HCl.

2.10.3 Electroelution of protein band from SDS gel

Following conventional SDS PAGE, the gel was stained with 0.1% Coomassie Blue (Sigma) in fixative (10% acetic acid; 30% methanol) and then destained in fixative, each for 10 minutes. The required band was excised, eluted in dialysis tubing using a Minigel apparatus (Biorad) and dialysed as described by McDonald *et al.* (1986), then lyophilysed.

2.10.4 Isoelectric focusing

Isoelectric focusing was carried out using a Pharmacia Phastgel IEF3-9, on the Pharmacia Phast protein electrophoresis system, and developed using a Pharmacia Phast Silver Stain kit as described by the manufacturer.

2.10.5 Excision of protein for antigen preparation

Following standard SDS PAGE, the gel was developed in ice cold 0.1M KCl for 10 minutes, allowing visualisation of proteins as white bands: the required band was cut from the gel; air dried for two hours; then homogenised in minimum volume phosphate buffered saline. This protocol was adapted from one described by Ausubel *et al.* 1987.

2.11 WESTERN BLOTS AND ANTIBODIES

2.11.1 Antigen preparation

To CelV prepared in phosphate buffered saline as described above was added an equal volume of Freund's Adjuvant (Sigma), Complete for the initial immunisation; Incomplete for the following boosts.

2.11.2 Inoculation and bleeding of rabbit

Approximately 0.5mg of CelV was used for each immunisation. A New Zealand red rabbit was immunised subcutaneously using a programme described by Ausubel *et al.* (1987): Following the initial immunisation, a booster immunisation was administered 28 and 42 days after this. A bleed was carried out 10 days after each immunisation, since this is when the response can be expected to be at a maximum. A sample of 5 to 10ml was taken, by bleeding from the ear. This was left to stand for several hours, the clot removed, and the serum centrifuged to remove debris, then stored in small aliquots at -20°C.

2.11.3 Purification of serum

Non-specific antibodies were removed from sera by immuno-absorption against an *E. coli* lysate, as described by Sambrook *et al.* (1989). A 100ml culture of K38 + pGP1-2 (see section 2.9.4) was grown to saturation in LB, then centrifuged and resuspended in 5ml 50mM Tris.Cl (pH8), 10mM EDTA (pH8), then freeze-thawed at least 4 times before sonication for six periods of 20 seconds and centrifugation

to remove cell debris.

The serum was diluted 1:10 in TNT (10mM Tris.HCl, pH8; 150mM NaCl; 0.05% Tween 20; 1% gelatin; 3% bovine serum albumin). To this 0.5 volume cell lysate was added, and the mixture was incubated at room temperature for 4 hours. The serum was then stored at 4°C in the presence of 0.05% sodium azide (Sigma).

2.11.4 Sample preparation

Strains were grown in LB to stationary phase (A_{600} 1.2-1.4) and used to prepare supernatant, periplasmic and cytoplasmic; or supernatant and cell samples (section 2.2.4.). Supernatant and sonicate samples were concentrated using trichloroacetic acid precipitation (Py *et al.*, 1991b), resuspended in 10% of the original volume of water and boiled for 10 minutes with a half volume of TSTD (Py *et al.*, 1991b), then chilled briefly on ice.

2.11.5 Electrophoresis and transfer

SDS polyacrylamide gel electrophoresis was carried out using a Phast System (Pharmacia). Pre-made 10-15% gradient gels (Pharmacia) were run as described by the manufacturer. Proteins were transferred to nitrocellulose filter (Hybond, Amersham) using one of two methods.

In Marseille, gels were blotted using a horizontal transfer apparatus (Biolyon). The sandwich was composed as follows, from top to bottom: filter paper soaked in upper buffer:

gel; nitrocellulose; filter paper soaked in lower buffer.

Upper buffer: 50mM Tris
75mM Boric acid
0.1% SDS

Lower buffer: 50mM Tris
75mM Boric acid
20% methanol

This was blotted for 30 minutes at 24V, then the gel was removed and treated as described below.

At Warwick, a Phast Transfer apparatus (Pharmacia) was used. The gel and nitrocellulose were sandwiched in the same orientation between 2 x 3 sheets of Phast Transfer filter papers, soaked in transfer buffer. Transfer was for 15 minutes, at 25mA, as described by the manufacturer, then the filter was removed and treated as described below.

2.11.6 Western analysis

The Western protocol was as follows (F. Barras and co-workers, pers. com.). The nitrocellulose filter was washed in 10ml polyvinylpyrrolidone (Sigma) in solution A for 15 minutes. Following 4-5 washes in Tween + solution A, it was incubated for 1 hour with the primary antibody in 10ml Tween + solution A (the original anti-CelV antiserum was diluted 1:1,000; the purified serum, owing to dilution during the protocol was diluted 1:50; the anti-Fel and Feh antibodies were diluted 1:50 as a result of their weakness). Following

a further 4-5 washes in Tween + solution A, the filter was incubated with the secondary antibody diluted 1:5,000 in 10ml Tween + solution A (alkaline phosphatase conjugated to anti-rabbit IgG for the anti-CelV antibodies; and anti-mouse IgG for the anti-Pel and anti-Peh antibodies, both from Promega). Following 4-5 further washes in Tween + solution A, 10ml developing solution was added and the filter was incubated until blue bands became visible to an acceptable intensity, and the reaction then stopped by the replacement of this with solution B.

AP buffer: 100mM Tris. HCl, pH8.5
100mM NaCl
5mM $MgCl_2$

Solution A: 25mM Tris. HCl, pH7.5
1mM $CaCl_2$
5mM $MgCl_2$
200mM NaCl

Tween was added to 0.05% final concentration. To reduce non-specific binding, Tween was substituted with Triton X 100, at the same concentration.

Solution B: 25mM Tris. HCl, pH7.5
1mM EDTA
200mM NaCl

Developing solution:

10ml AP buffer

33µl Nitroblue tetrazolium, 10% in 70% DMF

33µl 5-bromo-4-chloro-3-indolyl phosphatase, 5% in 100% DMF

2.12 POLYMERASE CHAIN REACTION

The reaction used was based on that of Ausubel *et al.* (1987). The reaction volume was 100µl, and consisted of the following:

0-9mM $MgCl_2$ (optimised for maximum full length yield)
8ul 10 x Taq polymerase buffer without Mg (Promega)
8ul 2mM dNTP mix
0.8ul 50µM oligonucleotide 1
0.8ul 50µM oligonucleotide 2
544ul 15µg/ml template DNA
1ul 2.5U/µl Taq polymerase (Promega)

The conditions used, on a Combi Thermal Reactor 2 (Hybaid) were as follows:

First step: 5 minutes, 94°C
Then 30 cycles of: 1 minute, 94°C
2 minutes, 50°C
3 minutes, 74°C

2.13 VIRULENCE ASSAYS

The strains to be assessed were grown to mid-stationary

phase in Luria Broth. Potatoes were washed and surface sterilised by soaking for 10 minutes in 5% hypochlorite solution, rinsed in tap water for 10 minutes and air dried. Sterile Gilson Pipetteman tips (0-200µl size) were used to make uniform bores in the tubers, and these were inoculated with 10µl samples of either an overnight culture (approximately 10^7 cells) or a 10^{-4} dilution of this (approximately 10^3 cells). The site of inoculation was sealed with High Vacuum Silicone Grease (BDH), and the tuber wrapped in alternate layers of wet paper towel and cling film, then incubated in a sealed plastic box at 25°C for 24, 48, 72 or 96 hours (adapted from Murata *et al* 1990 and Reeves 1992).

2.14 STRAINS AND PLASMIDS

Strains and plasmids used in this study are given in table 2.5.

Strain/ Plasmid	Characteristics	Source	Reference
E. coli			
JM101	delta(*lac-pro*), *supE, thi*, F' *traD36 proAB, lalq*, ZdeltaM15	J. Hinton	Yanisch-Perron *et al.*, 1985
TG1	JM101 *ecoK* derivative	J. Hinton	
K38	K12, HfrC	S. Tabor	Tabor and Richardson 1985
DH1	F$^-$, *recA1, endA1, thi-1, hsdR17, gyrA96 supE44, relA1*	D. Gill	Maniatis 1982
LE392	F$^-$, *hsdR514, supE44 supF58, lacY1, galK2 galT22, metB1, trpR55*	J. Hinton	Maniatis 1982
Ecc			
SCRI193	Wild type	J. Hinton	Forbes and Perombelon 1985
HC131	SCRI193+pHCP2	J. Hinton	Salmond *et al.*, 1986
GS7000	SCRI193 Cel$^-$	P. Reeves	Reeves 1992
GS7002	SCRI193 Cel$^-$	P. Reeves	Reeves 1992
DW102	HC131::Tn5		This study
DW306	GS7000+pDEE339 Ces$^-$		This study
HA9	GS7000+pDEE339 Ces$^-$		This study
HA10	GS7000+pDEE339 Cps$^-$		This study
HA21	GS7000+pDEE339 Cps$^-$		This study
HA42	GS7000+pDEE339 Ces$^-$		This study
HA59	GS7000+pDEE339 Ces$^-$		This study

Table 2.5 Strains and plasmids.

Ces$^-$ denotes mutants deficient in cellulase secretion; Cps$^-$ denotes mutants deficient in cellulase and pectinase secretion; Cam^R denotes chloramphenicol resistance.

Strain/ Plasmid	Characteristics	Source	Reference
HA61	GS7000+pDEE339 Cps$^-$		This study
HA65	GS7000+pDEE339 Ces$^-$		This study
HA66	GS7000+pDEE339 Cps$^-$		This study
HA75	GS7000+pDEE339 Ces$^-$		This study
HA77	GS7000+pDEE339 Cps$^-$		This study
MD42A	HA42 suppressor		This study
MD42B	HA42 suppressor		This study
MD42C	HA42 suppressor		This study
MD10J	HA10 suppressor		This study
MD21F	HA21 suppressor		This study
MD21M	HA21 suppressor		This study
JNH1	HC131::Tn5	J.N. Housby	Housby 1993
Plasmids			
pBR322	cloning vector	Amersham	Bolivar *et al* 1977
pV1C626	pBR322::*celV*	V. Cooper	Cooper 1992
pHCP2	pBR322::*lamB*	J. Hinton	Clement *et al* 1982
pLG339	SC101 ori	D. Gill	Stoker *et al* 1982
pDEE339	pLG339::*celV*		This study
pDW306	pDEE339, mutant derivative, Ces$^-$		This study
pHA9-pHA77	pDEE339, mutant derivative, Ces$^-$		This study
pUC19	cloning vector	D. Hodgson	Yanisch-Perron *et al* 1985
pUCV-1	pUC19::*celV*		This study

Table 2.5, continued.

Strain/ Plasmid	Characteristics	Source	Reference
pUCV9-pUCV306	pUCV-1, mutant derivatives, Ces$^-$		This study
pGP1-2	T7 polymerase	S. Tabor	Tabor and Richardson 1985
pT7-6	T7010 promoter	S. Tabor	Tabor and Richardson 1985
pT7V-1	pT7-6::*celV*		This study
pT7V9-pT7V306	pT7V-1, mutant derivatives, Ces$^-$		This study
pBluescript SK-	cloning vector		Stratagene
pSVC-1	pBluescript::*celV*		This study
pMD42A	pHA42, suppressing derivative		This study
pMD42B	pHA42, suppressing derivative		This study
pMD42C	pHA42, suppressing derivative		This study
pMD10J	pHA10, suppressing derivative		This study
pMD21F	pHA21, suppressing derivative		This study
pMD21M	pHA21, suppressing		This study
pDAH330	pIC19H::Cam^R	D. Hodgson	
pCelV2	pDEE339 derivative *Nae*I/*Eco*RI deletion		This study
pCelV3	pDEE339 derivative *Acc*I/*Eco*RI deletion		This study
pCelV4	pDEE339 derivative *Apa*LI/*Eco*RI deletion		This study
cHIL251-3	pSF6::*out* cluster	P.J. Reeves	Reeves *et al.*, 1993

Figure 2.5, continued.

CHAPTER 3

SEQUENCING OF THE OUT CLUSTER

3.1 Sequencing of the *out* cluster

As described in chapter 1, the *out* cluster of genes have been identified by cosmid complementation of Out mutants and subcloning down to a 12.7kb complementing fragment. Cloning and sequencing of the 13 genes, *outC-O* located on this fragment is described in Reeves *et al* (1993).

I participated in the random cloning into M13 of the three *EcoR*1 fragments which turned out to encode *outG* to *outO* (figure 3.1) and in the sequencing of the furthest downstream of these, a 1.2kb fragment which contains the majority of *outO*. As described in sections 2.4.1 to 2.4.5, the three *Eco*RI fragments were circularised and then sonicated, to produce random fragments, which were cloned into M13mp19, then sequenced using Universal primer, as described in section 2.4.8 and 2.4.9. The nucleotide sequence of the 1.2Kb downstream fragment is shown in figure 3.2, together with the predicted amino acid sequence of OutO. Sequence compilation and analysis was carried out by P. Reeves.

As discussed in chapter 1, and in Reeves *et al* (1993), OutO, by homology to PilD of *Pseudomonas aeruginosa*, is predicted to be a type 4 pilin peptidase, responsible for processing of several of the other Out proteins, which have amino terminal processing signals homologous to that of prepilin, PilA.

Figure 3.3 shows an alignment of OutO from Ecc with the

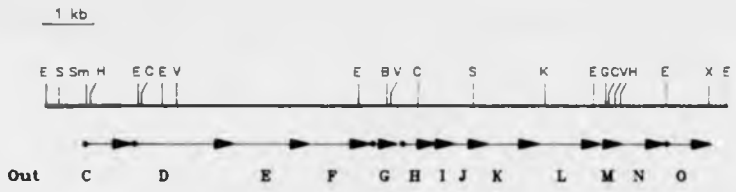


Figure 3.1. Restriction map of the Out cluster, showing the fragments sequenced.

The map shows the restriction sites in the 12.7Kb region carrying the genes *outC* to *outO*. Restriction endonuclease abbreviations: *Eco*RI(E), *Bam*HI(B), *Bgl*II(G), *Cla*I(C), *Hind*III(H), *Kpn*I(K), *Sma*I(Sm) and *Xmn*I(X).

Taken from Reeves *et al* (1993).

```
TTCATACCGTTTCTTTATCAACAATCAGGGACTAACGTGGACGATTTAAG
                                          V  D  D  L  R

GGAATTCGCACAGCTGTTTCCGGCGTGGTGGTTTGGGGCGCTGGGCGTGT
  E  F  A  Q  L  F  P  A  W  W  F  G  A  L  G  V

TGGGGTTGATTGTTGGCAGTTTTCTGAACGTGGTGATTTACCGCTTGCCG
L  G  L  I  V  G  S  F  L  N  V  V  I  Y  R  L  P

ATTATGCTGGAGCGTCGCTGGCGGCAGGACATCGAGCTTGAAACGGGCGT
 I  M  L  E  R  R  W  R  Q  D  I  E  L  E  T  G  V

GGCCGATCCCGATACGCGCTACAACCTGTGGTGGCCGCCTTCGTCTTGCC
  A  D  P  D  T  R  Y  N  L  W  W  P  P  S  S  C

CGCACTGCCAGCAGGCTATTGCGGTAAAAGATAATATCCCGCTATTCAGC
P  H  C  Q  Q  A  I  A  V  K  D  N  I  P  L  F  S

TGGCTGTGGCTGCGCGGCCGCTCCCGCTGCTGCCATCAATCGGTTTCTGT
 W  L  W  L  R  G  R  S  R  C  C  H  Q  S  V  S  V

GCAATARCCGTTAGTGGAAGTCATCACCATGCTGGCTTTTCTGGCGGCAG
  Q  Y  P  L  V  E  V  I  T  M  L  A  F  L  A  A

GTTTGCTCTGGTTGCCCGGCATGGCGCTGTGGGGGGCGTTGATTCTACTG
G  L  L  W  L  P  G  M  A  L  W  G  A  L  I  L  L

TCTTTCCTGTTGGTGCTGACCGTTATTGATATAAAAACGCTGCTGCTGCC
 S  F  L  L  V  L  T  V  I  D  I  K  T  L  L  L  P

GGATGAGCTTACGCTGTCGCTTCTGTGGATGGGGTTGCTGTTTAATCTGT
  D  E  L  T  L  S  L  L  W  M  G  L  L  F  N  L
```

Figure 3.2 Sequence of the *Eco*R1 fragment carrying *outO*, and the predicted amino acid sequence of OutO.

*Eco*R1 sites are underlined; termination codons are indicated by *.

```
CGGGGACATTTGTCTCATTGAATGATGCCGTAGTCGGCGCGATGGCCGGT
S  G  T  F  V  S  L  N  D  A  V  V  G  A  M  A  G

TATTTATCTCTGTGGCTACTTTATTGGGCATTCAAATACGCAACGGGCAA
 Y  L  S  L  W  L  L  Y  W  A  F  K  Y  A  T  G  K

AGAGGCGCTGGGGTACGGTGATTTTAAGTTGCTGGCTGCGCTGGGGGCTT
   E  A  L  G  Y  G  D  F  K  L  L  A  A  L  G  A

GGTTGGGCTGGCAGGCATTGCCGAATCTGGTTTTGGTGGCGGCGCTGAGC
W  L  G  W  Q  A  L  P  N  L  V  L  V  A  A  L  S

GGGCTGGTCGTGACTCTCATCTGGCGCGGGTTGCGTAAAGAGGATACCGC
 G  L  V  V  T  L  I  W  R  G  L  R  K  E  D  T  A

TAAACCGCTGGCCTTTGGCCCTGGCTGGCAATCGGCGGGGTATTCGGCAT
   K  P  L  A  F  G  P  G  W  Q  S  A  G  Y  S  A

GATAATGAACGGATTCAATCTGTAGAGAATGACAAAGGCAGTACGAATTA
*  *  *

TCGGCTGAATAATCGTCGGCTGAACATAGATCGGCAGGCATAAAAATAGG

CTGAGGGTATAAATACCATCAGCCTTTTTTATCTTTATGTGTGTGCCCTT

CAATGAATAAATCTTTGCCGGGCAAATGACAGGTTATTCTACCATCAGCA

TAAAGGTACTCACCCAAACGACTAAACAGAATGAGATACCCATAAAAAAA

TACTTCACCGATTTATTCTCCAGCAGAAACCACGCCAGCAACGATCCCGA

TTGTGCGTTTTATTATGGAAGGAAATAATTACGCTGCCGGTGATGTCACC

GAAGGTAATCTTTCCCGTTGTCATCACCAGAGTCACACCAGTTATGACGG

GATCGGCGCTAAATCTACGCCCAGTGAGGGGGGGAATTC
```

Figure 3.2, continued.

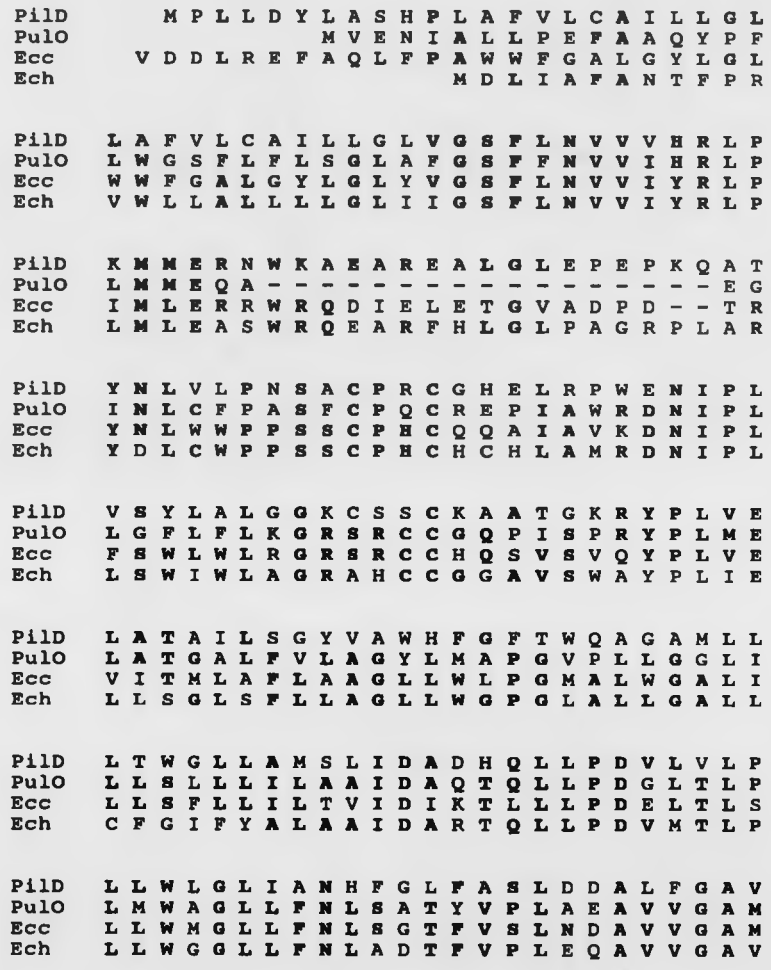


Figure 3.3 Alignment of the amino acid sequence of OutO with several homologous proteins.

Ecc denotes OutO of *Erwinia carotovora* subspecies *carotovora* (this study); Ech denotes OutO of *Erwinia chrysanthemi* (Lindberg and Collmer 1992); PilD is from *Pseudomonas aeruginosa* (Nunn *et al.*, 1990); PulO is from *Klebsiella oxytoca* (Pugsley and Reyss 1990). Amino acids exhibiting identity are shown in bold type. Sequences were aligned as described by Reeves *et al.*, (1993).

```
PilD   F G Y L S L W S V F W L F K L V T G K E G M G Y G
PulO   A G Y L S L W S V Y W V F R L L S G K E A L G Y Y
Ecc    A G Y L S L W L L Y W A F K Y A T G K E A L G Y G
Ech    A G Y L S L M L I Y W A F R L L S G R E A L G H G

PilD   D F K L L A M L G A W G G W Q I L P L T I L L S S
PulO   D F K L L A A L G A W L G W Q A L P Q T I L L S S
Ecc    D F K L L A A L G A W L G W Q A L P N L V L V A A
Ech    D F K L L A A L G A W L G W Q A L P N L V L I A S

PilD   L V G A I L G V I M L R L R N A E S G I P I P F G
PulO   P A A
Ecc    L S G L V V T L I W R G L R K E D T A L P L A F G
Ech    L T G L T A T L L W G R I H R L S M Q Q P L A F G

PilD   P Y L A I A G W I A L L W G D Q I T R T Y L Q F A
Ecc    P G W Q S A G Y S A
Ech    P W L A V S G M G L V L N V L G G W S H

PilD   G F K
```

Figure 3.3, continued.

corresponding proteins from several other systems: OutO of Ech, PilD of *Pseudomonas aeruginosa*, and PulO of *Klebsiella oxytoca*. As the diagram shows, these proteins show a high level of homology, with regions of substantial amino acid identity throughout the proteins. This high level of identity is reflected by the interchangeability of function: several studies (e.g. Lauer *et al.*,1993, Dupuy *et al.*, 1992) have demonstrated that mutants defective in OutO homologues can be complemented by OutO homologues from other systems. Particularly well conserved are a large number of (hydrophobic) leucine residues, and several cysteine residues, predicted to form a metal binding site. There is also a long region of near identity near the carboxy terminus.

Various studies (e.g. Nunn *et al.*,1990; Bally *et al.*, 1991; Lindberg and Collmer 1992) have indicated that OutO homologues are predicted to be integral inner membrane proteins, composed of several membrane spanning regions, but hydrophobicity plots do not equivocally predict the exact positions of these. P. Reeves has carried out an extensive investigation of this question using the construction of progressive deletions from the carboxy terminus of OutO, fused to *B* lactamase (Reeves *et al* submitted). The technique uses the fact that *B* lactamase is inactive in the cytoplasm and active in the periplasm, so that the position of the *B* lactamase domain can be established, based on ampicillin resistance, and related to the position in OutO where the fusion is situated. This resulted in the prediction that OutO comprises 8 transmembrane domains, with the carboxy

terminal domain predicted to be located in the cytoplasm. There is also a considerable region located near the amino terminus which is predicted to be on the cytoplasmic side. This encompasses the 4 cysteine residues, seen in figure 3.3 to be conserved, which are predicted to form a metal binding site.

3.2 Discussion

In collaboration with other members of the laboratory (Reeves *et al.*, 1993), a 12.7Kb fragment, identified as encoding factors required for the translocation of Cel and Pel across the outer membrane, was sequenced. It carries 13 genes, *outC* to *outO*, the predicted protein products of which exhibit considerable similarity to those of secretion apparatuses in other species. Downstream of *outO* there is a sequence that is predicted to produce a stem loop, which is thought to terminate transcription. Therefore, it seems unlikely that further *out* genes exist downstream of the cluster. However, since introduction of the cluster into *E. coli* does not reconstitute translocation of cellulase or pectate lyase across the outer membrane, it seems likely that there are other factors involved, perhaps upstream or at another locus.

Comparison of the sequence of OutO with several corresponding components from other systems identified a high level of similarity, despite the diverse overall functions of the machineries they are part of. This is particularly true in the region of the active site, and the carboxy

terminus, where almost perfect identity is seen over considerable stretches, indicating functional conservation.

Sequencing of the *out* cluster allowed comparison with the secretion clusters of other organisms, for which, as discussed in chapter 1, there are clues as to the functions of several of the proteins, such as OutD, OutE and OutO. As figures 1.8 and 1.9 illustrate, sequencing also highlighted differences between the secretion clusters, in terms of varying levels of sequence homology, organisation of gene clusters, and the presence and absence of certain proteins. This is particularly relevant when considering the paradoxical situation of secretion in Ecc and Ech, where exoenzymes exhibiting considerable homologies are not secreted by the Out apparatus of the other species. Therefore, sequencing of the cluster of genes encoding the components of the secretory apparatus provides an important basis for determining the direction of further studies in all aspects of secretion. In addition, as chapter 9 will describe, the search for extragenic suppressors of secretion mutants of CelV will focus on the *out* cluster, so that prior characterisation of this will be an advantage.

CHAPTER 4

RAISING POLYCLONAL ANTIBODIES AGAINST CELV

4.1 Introduction

It is likely that secretion relies on recognition of the correct orientation of sites which are distant in linear sequence but brought together as a result of protein conformation, rather than relying on a discrete recognition domain composed of contiguous sequences. This means that since integrity of the active site is also dependent on overall conformation it is unlikely that catalytic and secretory requirements will be mutually exclusive. In other words, a mutation that results in a secretion defect is also likely to be defective in cellulase activity. The problem that this presents is that up to this point the only handle available on the presence, absence, or stability of CelV was measurement of catalytic activity. It was therefore important to raise polyclonal antibodies against CelV. The first stage, therefore, was purification of sufficient CelV to provide the necessary antigen.

4.2 Purification of CelV

4.2.1 HBS binding

Work by V. Cooper (1992), based on sequence homology of the cellulase binding domain of CelV to that of CelZ of *Clostridium stercorarium* (Jauris *et al.*, 1990) has shown that CelV binds to HBS cellulose (Serva, Heidleberg). Cellulose binding would be a useful property to apply to purification. EGZ, for example, can be purified in a single step using its ability to bind strongly and specifically to Avicel (Py *et al.*, 1991b).

In order to apply this binding ability to purification, the effect of NaCl and Tris.HCl washes (used in the Avicel binding protocol, Py *et al.*,1991b) was determined. 1.5ml of sonicated cell culture of DH1 + pVIC626, grown in LB, was treated as described in section 2.9.2, and at each washing stage the supernatant was assayed for cellulolytic activity using CMC well assays (section 2.2.5), incubated for 18 hours. The results are shown in table 4.1. Row 1 shows activity in the initial sonicated cell culture sample, before the HBS binding protocol. Although a significant quantity remains unbound (row 2, the supernatant following pelleting of the HBS), very little is removed by either of the washes (samples 3 and 4), and a significant amount is eluted with water (sample 5). However, when attempting to apply this to large scale purification, it was found that the conditions required for binding were very specific, and were disrupted, for example, by the use of spheroplasting buffer (section 2.2.4; to isolate the cellulase-rich periplasmic fraction) or by shaking at 4°C (to eliminate proteolytic activity). Therefore, the ability of CelV to bind to HBS cellulose is not very useful for the purpose of purification.

4.2.2 T7 expression

A 2kb *Sph1/EcoR1* fragment (appendix 1) containing *celV* was cloned into pT7-6 of the T7g10 overexpression system (figure 4.1), and the resulting plasmid introduced into *E. coli* K38 carrying the plasmid pGP1-2 (figure 4.2). As described in section 2.9.4, CelV was overproduced by induction at 42°C,

Sample	Relevant information	Halo diameter (mm)
1	Before HBS	14
2	After HBS	9
3	After Tris wash	7
4	After NaCl wash	6.5
5	After water wash	11

Table 4.1. HBS binding assay

Supernatant samples were taken at various stages during the HBS binding protocol (section 2.9.2). The table shows sample numbers; information about the stage at which they were taken; and the diameter of haloes on cellulase assay plates, following incubation of a 50ul sample for 18 hours at 37°C, in a well of 6mm.

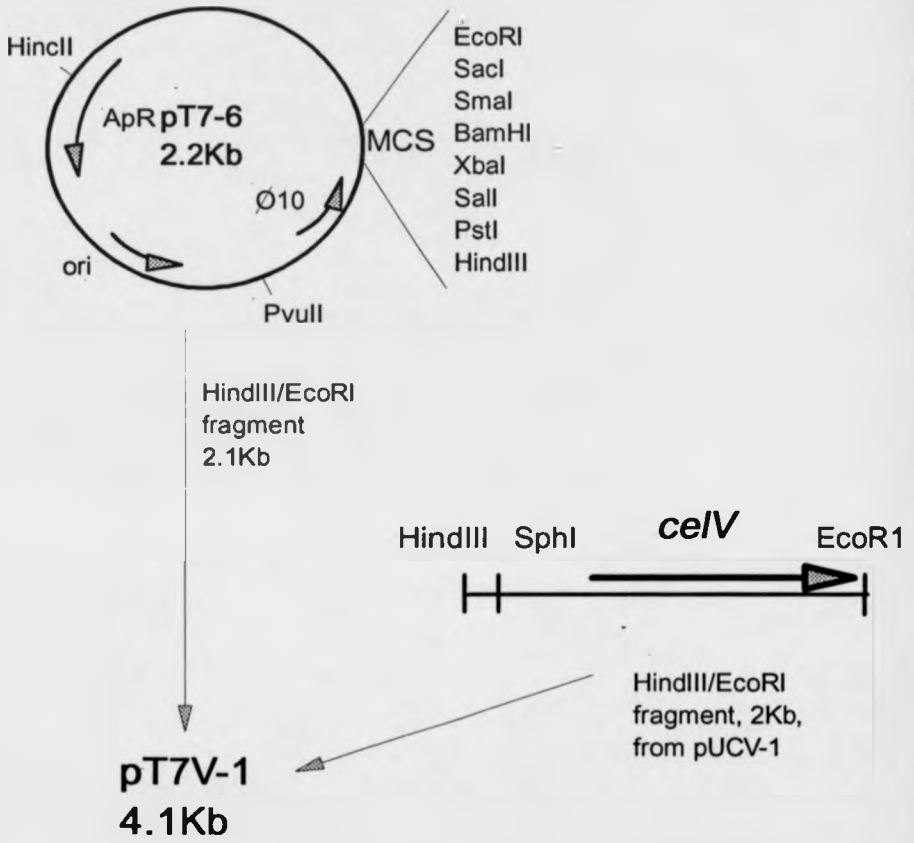
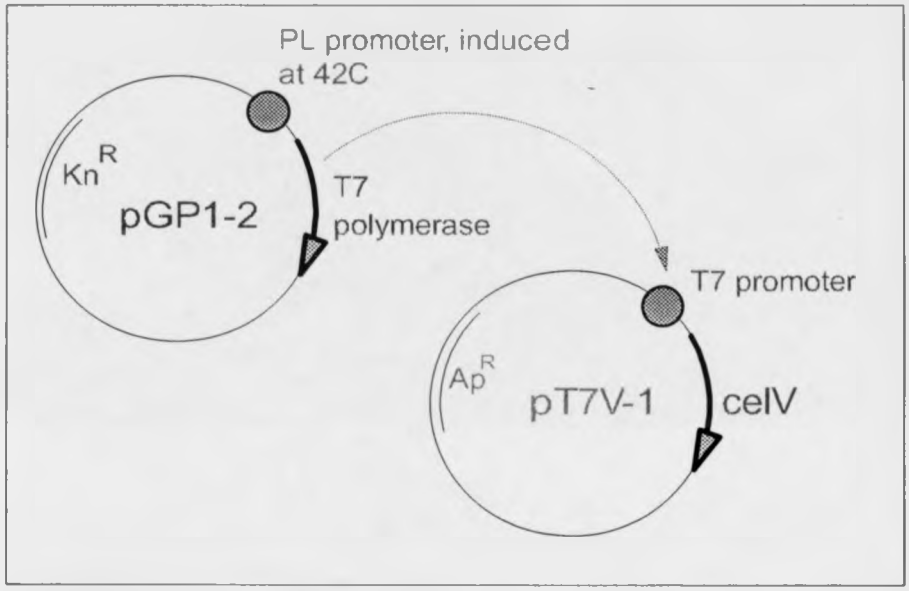


Figure 4.1. Cloning of *celV* into pT7-6.



K38

Figure 4.2. The T7 expression system (Tabor and Richardson 1985).

celV is cloned into pT7-6, downstream of the T7 promoter, while on pGP1-2 the gene encoding T7 polymerase is cloned downstream of a temperature sensitive promoter. Induction at 42°C results in switching on of this promoter, resulting in synthesis of T7 polymerase, which in turn results in high level expression of *celV*.

followed by incubation at 30°C for 0, 60, 90, 120 and 150 minutes.

Since CelV is not secreted by *E. coli*, accumulating instead in the periplasm, a periplasmic fraction was taken by spheroplasting (section 2.2.4). The samples were then assayed for protein content (section 2.9.1) and for cellulase activity, simply by measuring the halo size resulting from well assays on CMC plates incubated for 18 hours (section 2.2.5). As the results in table 4.2 show, cellulase activity levels were still relatively low, so that it was decided to concentrate the samples approximately four-fold using Amicon Centriprep (section 2.9.3), the assay results for which are also shown in table 4.2.

4.2.3 Identifying and isolating the correct band

The concentrated samples, together with, as controls, K38 and K38 + pVIC626 treated in the same way (expressed for 90 minutes), were prepared for SDS PAGE and electrophoresed as described in section 2.10.1. The resulting gel was treated to remove SDS, overlaid with a cellulase activity assay gel (section 2.10.2), and then Coomassie stained (section 2.10.1). The resulting gel and overlay are shown in figure 4.3.

In order to determine the position of CelV on the gel, the molecular weight standards were used to plot log of size against distance moved on the gel, as shown in figure 4.3. Since the predicted size of CelV is 50KDa, it would be predicted to move a distance of between 7 and 7.6cm. This

Strain	Induction time (minutes)	Before concentration		After concentration	
		Protein conc. (ug/ml)	Halo size (mm)	Protein conc. (ug/ml)	Halo size (mm)
K38	90	147	0	472	0
K38 + pVIC626	90	162	7	468	10
K38 + pGP1-2	0	120	8	550	14
+ pT7V-1	60	262	9	535	14
	90	180	12	524	18
	120	173	11	490	18
	150	190	12	520	17

Table 4.2. Protein content and cellulase activity of T7 expression samples, before and after concentration.

The table shows, from left to right, the strain used; the time of induction at 42°C; protein concentration, predicted using the Biorad assay; and the diameter of halo on a CMC plate, following incubation of a 50ul sample in a 6mm well at 37°C for 18 hours. Samples used were periplasmic fractions, before and after concentration using Amicon Centriprep.

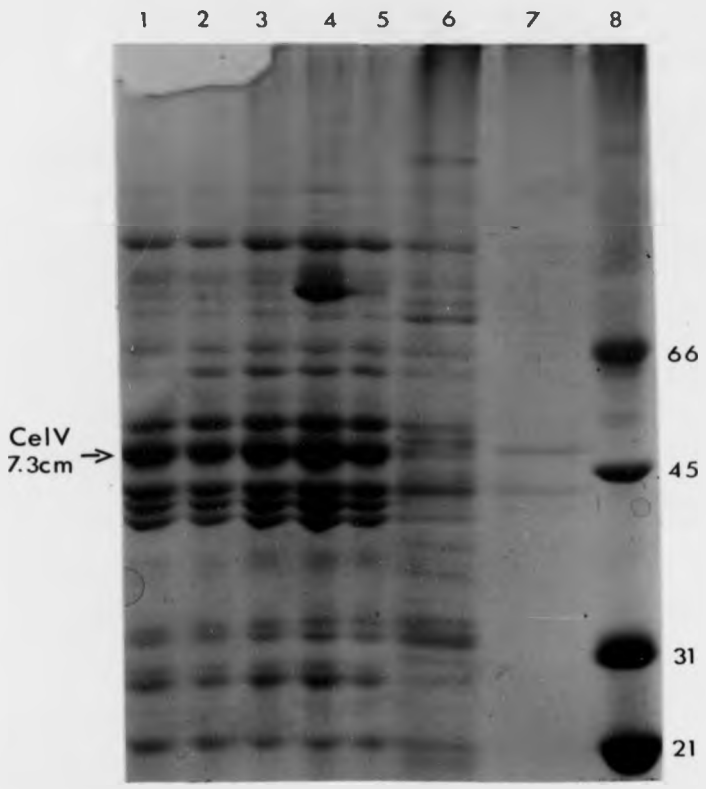
Figure 4.3. Analysis of T7 expression samples, and the identification of CelV.

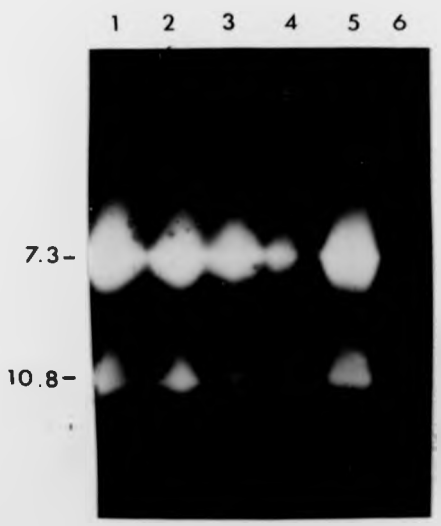
(A) shows SDS PAGE of concentrated periplasmic fractions from K38 + pGP1-2 + pT7V-1, following various expression conditions, described in detail in the text, with K38 and K38 + pVIC626 as controls. The gel has been Coomassie stained. Lanes: 1-5 = K38 + pGP1-2 + pT7V-1, induced at 42°C for 30 minutes (section 2.9.4), then allowed to express for various time periods at 30°C: 1 = no incubation at 30°C; 2 = 60 minutes; 3 = 90 minutes; 4 = 120 minutes; 5 = 150 minutes. 6 = K38 + pVIC626 and 7 = K38, both treated as described for the T7 samples, and allowed to express at 30°C for 90 minutes. Lane 8 shows molecular weight standards, the sizes of which are given on the right. The predicted position of CelV is indicated by an arrow on the left.

(B) shows a cellulase activity overlay from the gel shown in A. Lanes are numbered as already described for A.

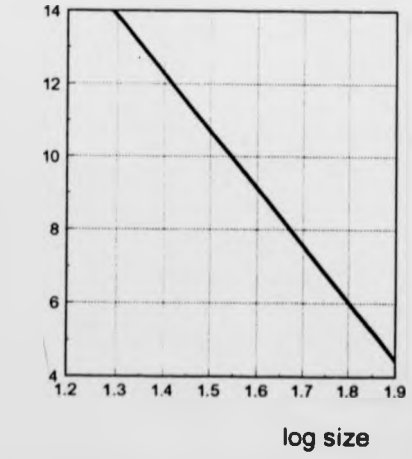
(C) shows a plot of log molecular weight against distance moved down the gel, for the molecular weight standards seen in A.

A


1 2 3 4 5 6 7 8
CelV
7.3cm
66
45
31
21


1 2 3 4 5 6
7.3-
10.8-

C


Distance
moved (cm)
14
12
10
8
6
4
1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9
log size

information was then compared with the cellulase overlay, on which a major band can be seen at 7.3cm, resulting in the identification of the band indicated on the SDS polyacrylamide gel as CelV.

Interestingly, another minor band is seen on the activity overlay, at 10.8cm, which would mean a predicted size of 32.36KDa, based on the graph. Based on the sequence of *celV* (appendix 1) the catalytic domain would be predicted to be approximately of this size (using the position of the first proline and threonine residues which form the linker region to indicate the end of the catalytic domain). The appearance of this activity band therefore coincides well with predictions about the functional independence of the two domains, which have already been demonstrated for EGZ of Ech (Py *et al.*,1991b).

It appears that there is little difference between the cellulase activity produced from the later time points of T7 expression. Therefore, 120 minutes was chosen for future experiments.

The information gained was used to prepare more of the 120 minute sample in the same way, and then load two entire SDS polyacrylamide gels as before. A small section was removed from one side of a gel and used to prepare an overlay, as before, while the remainder was briefly stained (section 2.10.3) and destained. The band corresponding to CelV was cut from the gels, then eluted, dialysed and lyophilised as described in section 2.10.3. The resulting sample was

analysed by isoelectric focusing (section 2.10.4) and the Phastgel developed in the Phast apparatus using a Silver Stain kit supplied by the manufacturer. Figure 4.4 shows the resulting gel, which includes isoelectric point standards in the range 4.75-10.6 (BDH). V. Cooper (1992) previously reported the appearance of two bands, at pI 4.4 and 4.6, and these are indeed clearly visible towards the top of the gel. However, one further band appears, further down the gel. In addition, the yield resulting from this protocol was low, being in the order of 1-10µg in the total 200µl sample resulting. This is perhaps as a result of interactions between cellulase and the dialysis tubing used.

In view of the ease of cutting a protein band from a gel compared with conventional purification techniques, and the ease with which antiserum can be affinity-purified, it was decided that the general technique was still a viable approach, despite the additional band apparent on the IEF gel. However, since the yield resulting from elution and dialysis was low, a different approach was taken for the preparation of antigen. Large scale SDS PAGE of the T7 sample from 120 minutes expression was performed as before. The gel was then developed in potassium chloride and the relevant band excised and prepared for immunisation as described in sections 2.10.5 and 2.11.1.

4.3 Immunisation of rabbit

The homogenised gel slice containing CelV was prepared for immunisation of a New Zealand red rabbit as described in

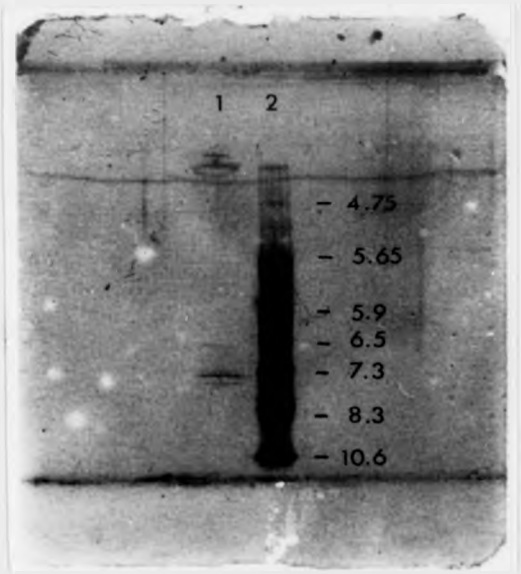


Figure 4.4. IEF gel of the purified CelV sample, eluted from a gel slice.

Lane 1 = CelV purification sample; lane 2 = isoelectric point standards, the values for which are given on the right.

section 2.11.2. Section 2.11.2 also describes the timetable of immunisations and bleeds which was followed, and which is illustrated in figure 4.5.

4.4 Analysis of serum

Rabbit serum resulting from the bleeds following the first and second boosts was first tested by dot blot analysis in comparison with serum taken from the rabbit prior to contact with the antigen.

Overnight cultures of GS7000 and TG1 with and without pVIC626 were spotted in 10ul samples onto nitrocellulose (Hybond, Amersham), allowed to dry for 30 minutes at room temperature, then incubated at 80°C for 30 minutes. The filter was then treated exactly as described for Western analysis (section 2.11.6). The results are shown in figure 4.6. As the blot shows, the antiserum from the pre-immunisation bleed fails to react with either TG1 or GS7000, regardless of the presence of CelV, as expected. The antisera from the bleeds taken after the first and second boost inoculations react with both strains. They react more strongly with TG1 + pVIC626 than with GS7000 + pVIC626. Although there is evidence that there may be a small reaction with each of these strains in the absence of CelV, the reaction is much reduced, and therefore unlikely to cause a problem.

The purity of the serum was investigated further by Western analysis. Periplasmic fractions of the T7 expression

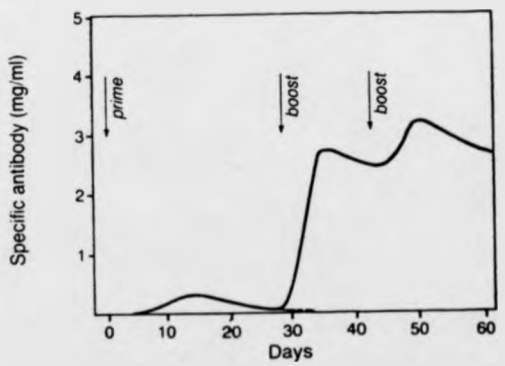


Figure 4.5. Programme for immunisation of rabbit.

The graph shows the recommended time interval between inoculations and bleeds, and the predicted antigenic response. From Ausubel *et al* (1987).

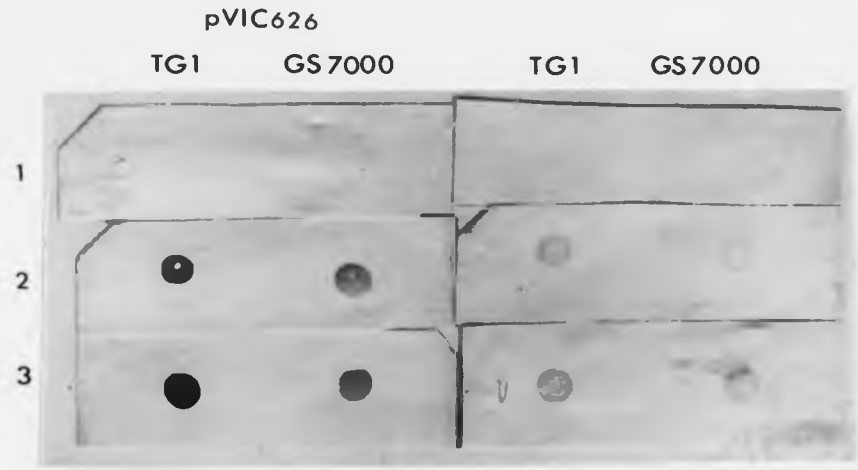


Figure 4.6. Dot bot ELISA analysis of serum samples.

Btrains used are given at the top. Serum samples are given on the left: 1 = serum sample taken prior to inoculation; 2 = serum sample taken following the first boost inoculation; 3 = serum sample taken following the second boost inoculation.

strain, K38 + pGP1-2 + pT7-6 with and without the *celV*-containing insert (figures 4.1 and 4.2) were subjected to SDS PAGE using a Phast 10-15 gradient gel (sections 2.11.4 and 2.11.5). Proteins were transferred to nitrocellulose (section 2.11.5) and analysed by Western blotting (section 2.11.6) using 10ul serum from the bleed following the second boost in 10ml solution A as the primary antibody. As figure 4.7 shows, there is evidence for non-specific binding to several proteins. At the predicted size of CelV, 50kDa, cross-reacting material appears in both lanes, though this is more pronounced in the lane where the T7 strain expressing CelV was loaded than in that in which pT7-6 contained no insert. This suggests that the serum cross-reacts with two proteins of 50kDa, one being CelV and the other being non-specific.

4.5 Affinity-purification of serum

The serum from the bleed following the second boost was purified by incubation with a lysed culture of the T7 strain that did not express CelV, as described in section 2.11.3. Lanes 3 and 4 of figure 4.7 show Western analysis of the T7 strains with and without the *celV* insert, using the purified serum. This resulted in the appearance of a single band, at the expected size of 50kDa, which was only present in the blot of the strain containing *celV*. In other words, affinity-purification successfully eliminated non-specific binding. All future experiments therefore used the affinity-purified serum.

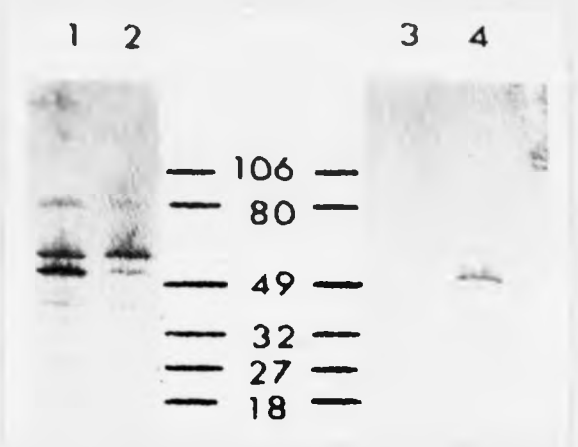


Figure 4.7. Western analysis of serum samples before and after affinity-purification.

Lanes 1 and 2 were subjected to Western analysis using serum from the bleed following the second booster inoculation. 1 = K38 + pGP1-2 + pT7V-1; 2 = K38 + pGP1-2. Lanes 3 and 4 were blotted using the same serum sample, following affinity purification. 3 = K38 + pGP1-2; 4 = K38 + pGP1-2 + pT7V-1. The T7 expression strains were induced and allowed to express for 90 minutes (section 2.9.4), and used to prepare periplasmic fractions. Molecular weight standards are given in the centre, in kDa.

4.6 Verification of the strength and specificity of the purified serum

It was important to ensure that the band seen in lane 4 of figure 4.7 was in fact CelV; that the serum did not exhibit any significant non-specific binding to *Erwinia* proteins; and that the antibodies were sufficiently strong in their recognition of CelV to be workable.

Supernatant, periplasm and cytoplasm fractions (section 2.2.4) were prepared for GS7000 and GS7000 + pDEE339 (see chapter 6 for construction of pDEE339 and reasons for its use), the supernatant and cytoplasm fractions were concentrated ten-fold (section 2.11.4) and all samples were boiled in TSTD (section 2.11.4) and subjected to SDS PAGE on a Phast gel (section 2.11.5) and then Western analysis (sections 2.11.5 and 2.11.6). The result is shown in figure 4.8. The band corresponding to CelV at 50kDa is clearly visible in the supernatant sample of GS7000 + pDEE339, while nothing is seen in GS7000. In both samples, some non-specific binding is seen in the cytoplasmic fractions, but since the proteins of importance in this study will localise to the periplasm or supernatant, this is not a problem. Also, as later Western analysis demonstrated, non-specific binding can be reduced by substituting Triton X 100 for Tween in the later washes of the Western protocol.

Specificity of binding was verified further by carrying out the same analysis using *Erwinia chrysanthemi*. pDEE339 was introduced by electroporation into E1007, a derivative of

Figure 4.8. Verification of serum specificity in Ecc GS7000.

Strains used are indicated at the top. C = cytoplasm (i.e. sonicate), P = periplasm, S = supernatant. Molecular weight standards are given on the left, in kDa.

the wild type strain *Ech* 3937 in which *celZ* is inactivated by insertion of an omega interposon in the middle of the signal sequence (Py *et al.*, 1993). As figure 4.8 shows, a single band of 50kDa is recognised in the presence of CelV, but not in its absence. The band corresponding to CelV is located exclusively in the periplasm, indicating that Ech is incapable of secreting CelV. This confirms the observation of Py *et al* (1991a) based on the localisation of catalytic activity.

4.7 Discussion

The overproduction of CelV using the T7 g10 system of Tabor and Richardson (1985), followed by excision of a gel slice identified as containing CelV resulted in purification of CelV in sufficient quantity and purity to allow production of polyclonal antibodies. Once affinity-purified, the polyclonal antiserum was shown to specifically bind CelV, without significant nonspecific binding.

Provided that fractionation samples were concentrated 10-fold prior to electrophoresis, the antiserum reaction was sufficiently strong to allow good recognition of CelV in single or low copy, establishing that the serum is amenable to the study in question, since this is based largely on low copy expression.

In addition, no cross-reaction was seen with EGZ or *Ecn* despite the high degree of homology in the catalytic domain. This reflects a similar finding with respect to CelV and anti-EGZ antisera (Barras and coworkers, pers. com.).

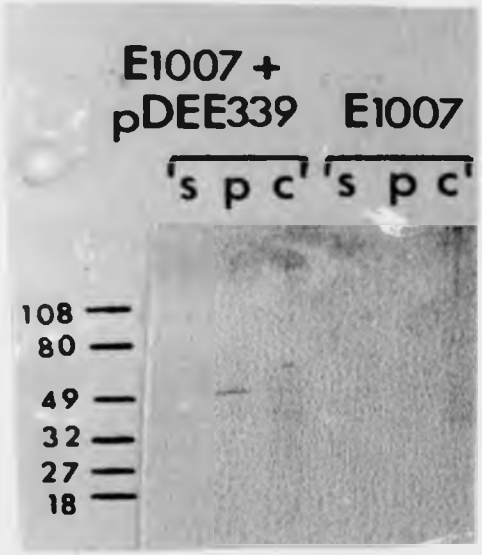


Figure 4.9. Verification of serum specificity in Ech E1007.

Western analysis using affinity-purified serum. Strains used are given across the top of the gel. S = supernatant; P = periplasm; C = cell sonicate (i.e. cytoplasm). Molecular weight standard positions are given on the left, in KDa.

CHAPTER 5

CHARACTERISATION OF GS7000, AND THE ROLE OF CELLULASE IN PATHOGENICITY

5.1 Introduction

The study of CelV required the expression of *celV* in a clean background which is cellulase minus. Since GS7000, the mutant used for this purpose was generated using randomised chemical mutagenesis it was important to ensure that the mutation was in the *celV* locus itself, and not in some as yet uncharacterised regulatory locus elsewhere.

In addition, it was decided to use this mutant to investigate the effect of cellulase deficiency on the ability of Ecc to cause soft rot disease of potato tuber. Pectate lyases have been demonstrated to be extremely important determinants of the ability of *Erwinia* species to cause soft rot (Hinton *et al.*,1989b; Collmer and Keen 1986). Indeed, expression of Pels by *E. coli* (Zink and Chatterjee 1985) or inoculation with acellular enzyme preparations (Barras *et al.*,1987) have been demonstrated to result in rot of potato tubers. It therefore seemed to be the consensus view that the other classes of secreted enzymes play no role or very small roles in virulence, with the result that much of the research carried out on *Erwinia* species concentrates on pectate lyases. This is despite the lack of firm evidence against a pathogenic role for cellulases or proteases.

Cellulases and proteases have been cloned from various phytopathogenic bacteria in addition to *Erwinia* spp., such as *Xanthomonas* spp. and *Pseudomonas* spp. In *Xanthomonas campestris* p.v. *campestris*, both cellulases (Gough *et al.*, 1988) and protease (Dow *et al.*, 1990) have been demonstrated

to exert slight effects on virulence, while the major endoglucanase of *Pseudomonas solanacearum* plays a significant role in pathogenesis of tomatoes (Roberts *et al.*, 1988). Concerning *Erwinia* spp. there have been several studies of the role of cellulase or protease in virulence, although conclusions vary. Dahler *et al.* (1990) have demonstrated that mutants defective in one or all of the proteases of *Ech* were not impaired in virulence, as determined using potato disk assays or inoculation of chrysanthemum plant stems. Conversely, Aymeric *et al.* (1989) have presented evidence that the absence of EGZ has a profound effect on the virulence of *Ecn* on *Saintpaulia*. However, the marker exchange mutant used in this study has since been shown to be deficient in the secretion of cellulase and pectinase (Py *et al.*, 1993; F. Barras, pers. com.). This suggests that the strain possesses an additional mutation in the *out* cluster, which would itself have a significant effect on virulence.

The aim of this study, then, was to investigate the effect of cellulase deficiency on the ability to cause soft rot, in order to determine unequivocally whether or not this enzyme is a significant virulence factor.

5.2 Characterisation of GS7000

GS7000 was isolated on the basis of its Cel$^-$, Pel$^+$, Prt$^+$ phenotype on enzyme detection plates, following EMS (methane sulphonic acid ethyl ester) mutagenesis by P. Reeves (Reeves 1992).

Figure 5.1 shows the results of cellulase activity assays of cytoplasmic, periplasmic and supernatant fractions from GS7000 and the wild type, SCRI193. The results indicate that this cellulase minus phenotype is not due to accumulation of cellulase activity. In addition, other extracellular enzymes (pectate lyase and protease) are synthesised and secreted normally (Reeves 1992).

Ideally, both the investigation of the role of cellulase in virulence and the use of a cellulase mutant as a background for secretion studies would be best carried out using a marker exchange mutant, in which *celV* is interrupted, so that the precise nature of the mutation responsible for the phenotype is known. However, marker exchange has proved difficult at the *celV* locus in strain SCRI193, so that it was necessary instead to use GS7000. Efforts were therefore made to ensure that the mutation responsible for the observed phenotype resided in the *celV* locus and affected only the synthesis or activity of cellulase.

The genetic nature of GS7000 was investigated by complementation using a pSF6-based cosmid library of SCRI193 DNA packaged into lambda. Nine out of one thousand transductants exhibited cellulase activity when screened on cellulase detection plates, and cosmids pSFD1-9 were prepared from these, analysed by gel electrophoresis and introduced into *E. coli* TG1. All nine of the cosmids encoded cellulase activity when expressed in *E. coli*, and, as figure 5.2 shows for five of these, showed similarities in the restriction fragments seen when digested with EcoR1. Several of these

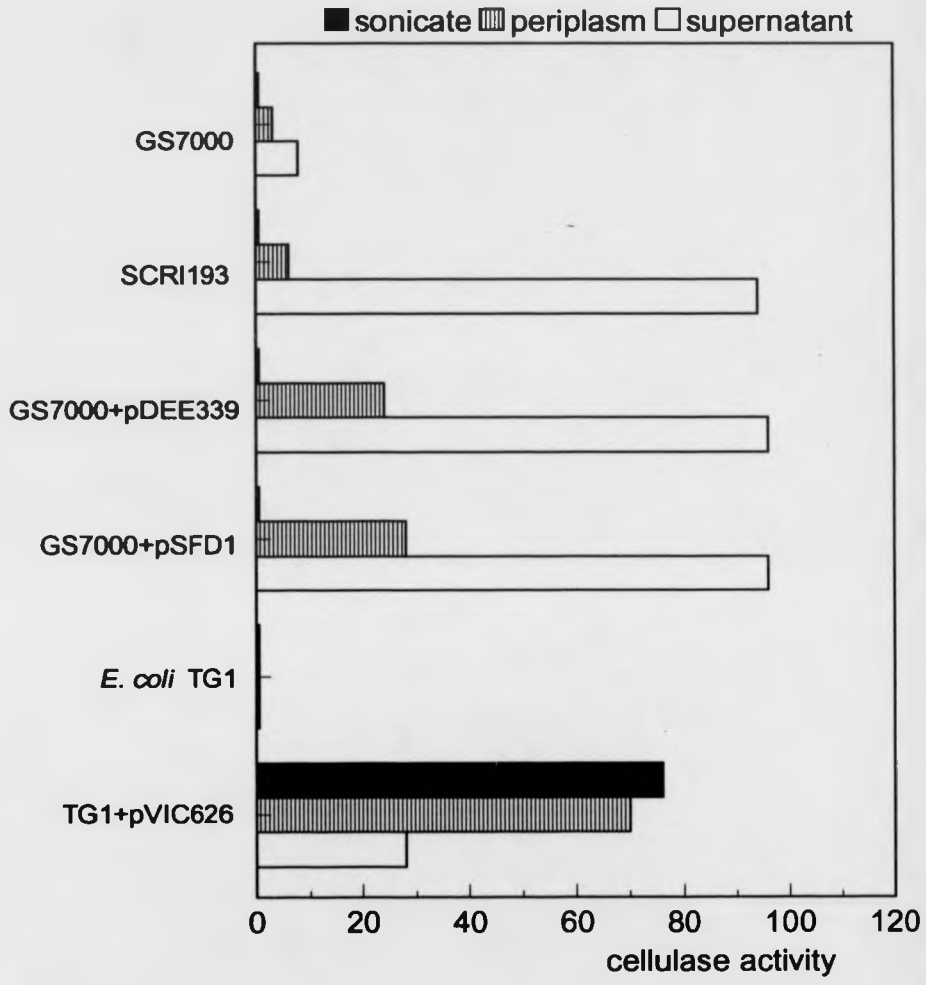


Figure 5.1. Cellulase assays of strains used in the virulence study.

Sonicate, periplasm and supernatant fractions (section 2.2.4) were assayed (section 2.2.5). Results are expressed as percentage SCRI193 total activity, based on a standard curve constructed using serial dilutions of SCRI193 whole culture sonicate.

Fractionation control: at least 89% *B* galactosidase activity is located in the sonicate.

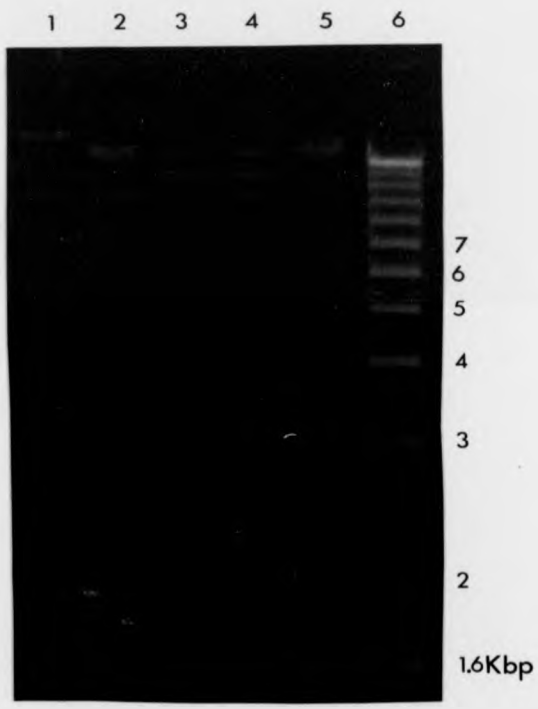


Figure 5.2. Cosmids complementing GS7000.

The photo shows agarose gel electrophoresis of cosmids pSFD1 to pSFD5, indicated by the numbers heading the lanes, cut with EcoR1. Lane 6 is 1Kb Ladder (BRL), and the molecular weights of these standards are indicated on the right.

fragments, in particular, fragments of 9.4, 5.2 and 2.9Kb, were in common with cosmids characterised during the cloning of *celV* (Cooper 1992) indicating the presence of the structural gene. This provides circumstantial evidence that GS7000 can only be complemented by the structural gene and therefore that the mutation responsible maps to the *celV* locus.

5.3 Transduction analysis

The question as to whether the phenotype of GS7000 results solely from a mutation within *celV* was investigated by transduction analysis. First, a derivative of SCRI193 was constructed in which *celV* was linked to Tn*5*, encoding kanamycin resistance. HC131, a derivative of SCRI193 which contains pHCP2, was used because this plasmid contains the *E. coli lamB* gene. This encodes the surface receptor for lambda, allowing lambda to infect, but since it is unable to replicate within *Erwinia* spp., it acts as a suicide vector (Salmond *et al* 1986). Therefore, on infection of HC131 with a high titre lysate of lambda::Tn*5* (section 2.6), the inability of lambda to replicate means that kanamycin resistant transductants will only result if the transposon hops into the chromosome, which it does at random sites. Transductants were pooled and used to prepare a heterogeneous culture in LB, Mg (section 2.1), which was infected with the generalised transducing phage phiKP to produce a high titre lysate (section 2.7.1). This was then used to transduce cellulase minus mutants, GS7000 and GS7002, and linkage of *celV* to Tn*5* was identified by looking at

cotransduction of these. In other words, transductants were selected on the basis of kanamycin resistance and then picked onto CMC assay plates to identify cellulase plus colonies. These were investigated further by preparing a phiKP lysate of the strain in question, transducing cellulase deficient mutants, and then looking at the frequency with which cellulase activity and kanamycin resistance were cotransduced. This resulted in the isolation of strain DW102, which exhibited cotransduction frequencies of 44 and 62% for transduction into GS7002 and GS7000, respectively. Of the two classes of kanamycin resistant transductants, those which were Cel$^+$ and those which were Cel$^-$ had precisely the same phenotype as HC131 and GS7000, respectively. Figure 5.3 shows that cellulase synthesis and secretion followed the same pattern. In addition, growth rate, pectate lyase activity and protease activity (as measured on assay plates) were identical.

These data support the complementation data, providing evidence that the mutation responsible is in the *celV* locus. The clean reversion of the phenotype in all respects in Cel$^+$ transductants provides circumstantial evidence against the existence of a second site mutation (at least one which would interfere with the use of GS7000 in these studies), or a regulatory mutation with pleiotropic effects.

5.4 Western analysis of GS7000 and complemented derivatives

Figure 5.4 shows Western analysis of GS7000 using the purified polyclonal antibodies against CelV. This failed to

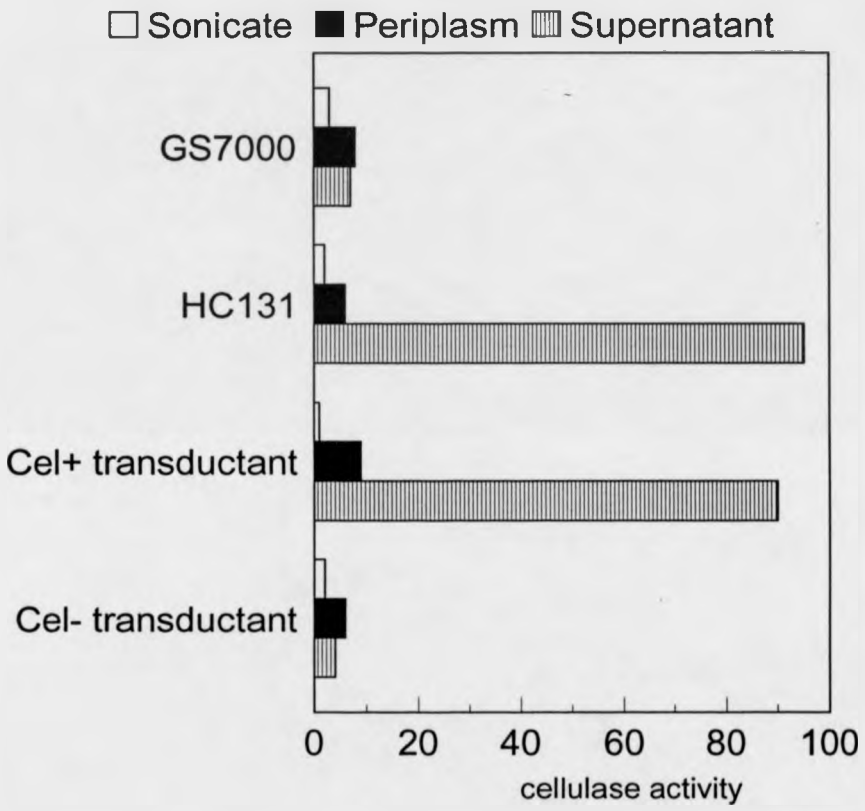


Figure 5.3. Cellulase assays of transductants.

As described in the text, a phiKP lysate was made on strain DW102, and used to transduce GS7000. Examples are taken from the Cel^+ and Cel^- populations, and cell fractions (section 2.2.4) assayed for cellulase activity (section 2.2.5). Activity is expressed as percentage SCR1193 total activity, based on a standard curve of serial dilutions of whole culture sonicate.

Fractionation control: at least 91% *B* galactosidase activity is located in the sonicate.

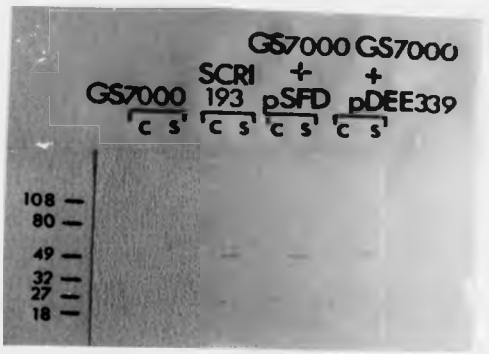


Figure 5.4. Western analysis of GS7000 and complemented derivatives.

Using polyclonal antibodies against CelV (sections 2.11.4 to 2.11.6). The figure shows a comparison of GS7000 with the wildtype, SCR1193, and GS7000 complemented with pDEE339 and pSFD1. S = supernatant; C = cell sonicate (i.e. periplasm plus cytoplasm). Positions of molecular weight standards are shown in KDa on the left.

recognise either a full size product or a degradation or truncation derivative. The most obvious explanation of this is either that GS7000 is a regulatory mutant which does not synthesise CelV, or that a nonsense or missense mutation results in either a truncated or an unstable form that fails to be recognised by the antibodies. Figure 5.4 shows that when GS7000 is complemented by either pSFD1 or pDEE339 (see chapter 6 for construction of pDEE339), CelV is synthesised and secreted to the same extent as in the wild type SCRI193.

5.5 The role of cellulase in virulence

The importance of CelV in soft rot was investigated by comparison of the ability of GS7000 and SCRI193 to macerate potato tuber tissue, as described in section 2.13 Tubers were inoculated with cell suspensions in stab holes, and the stab sites then sealed to increase anaerobiosis, and the potatoes incubated wrapped in alternate layers of wet paper towel and cling film, to increase humidity. The conditions used therefore aim to closely imitate those under which *Erwinia* species attack potato tubers in storage, i.e. high humidity and low oxygen potential.

As figures 5.5 and 5.6 show, GS7000 is significantly reduced in its ability to cause soft rot compared with SCRI193. This deficiency is restored by complementation either with pDEE339 or pSFD1, although in some cases (particularly the lower inoculum of GS7000 + pSFD1) restoration is not completely to wild type levels. This is despite the observation from figure 5.1 that cellulase activity is

Figure 5.5. Virulence assays on potato tubers.

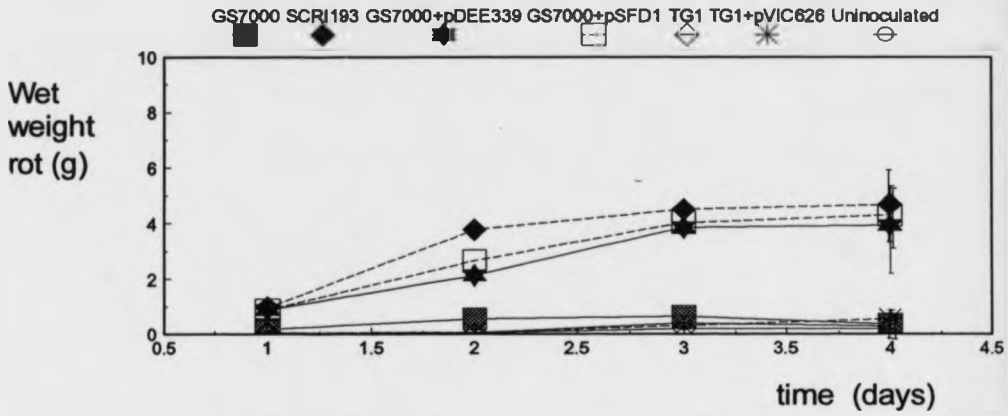
Cyprus New potatoes were inoculated with liquid cultures and incubated for up to 4 days, at which time macerated tissue was removed and weighed. Results shown are means for 6 replicas of each strain, for each time point and each inoculum size.

(A) shows the results for inoculation with approximately 10^7 cfu.

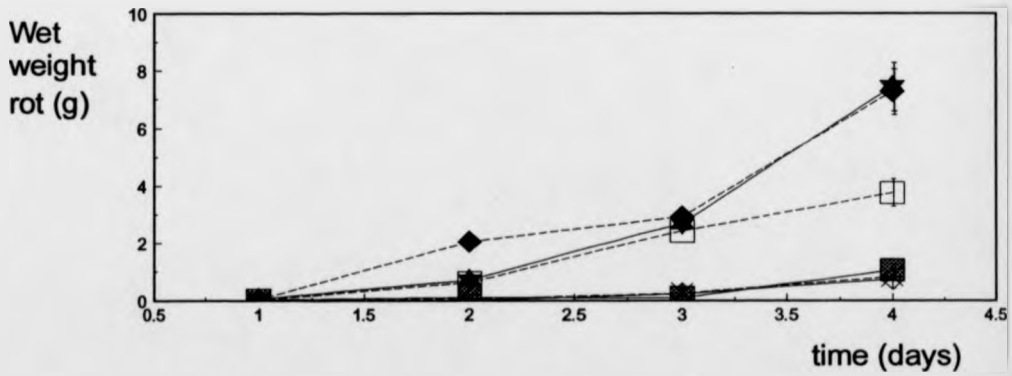
(B) shows those for approximately 10^3 cfu.

For simplicity, 95% confidence bars are given only for the final time point, at which point they can be assumed to be at their widest. Similar effects were seen for other New potato cultivars; Cara baking potatoes; and Romano Red, although average rot levels varied considerably.

A


GS7000 SCRI193 GS7000+pDEE339 GS7000+pSFD1 TG1 TG1+pVIC626 Uninoculated
Wet weight rot (g)
0
2
4
6
8
10
0.5
1
1.5
2
2.5
3
3.5
4
4.5
time (days)

B


Wet weight rot (g)
0
2
4
6
8
10
0.5
1
1.5
2
2.5
3
3.5
4
4.5
time (days)

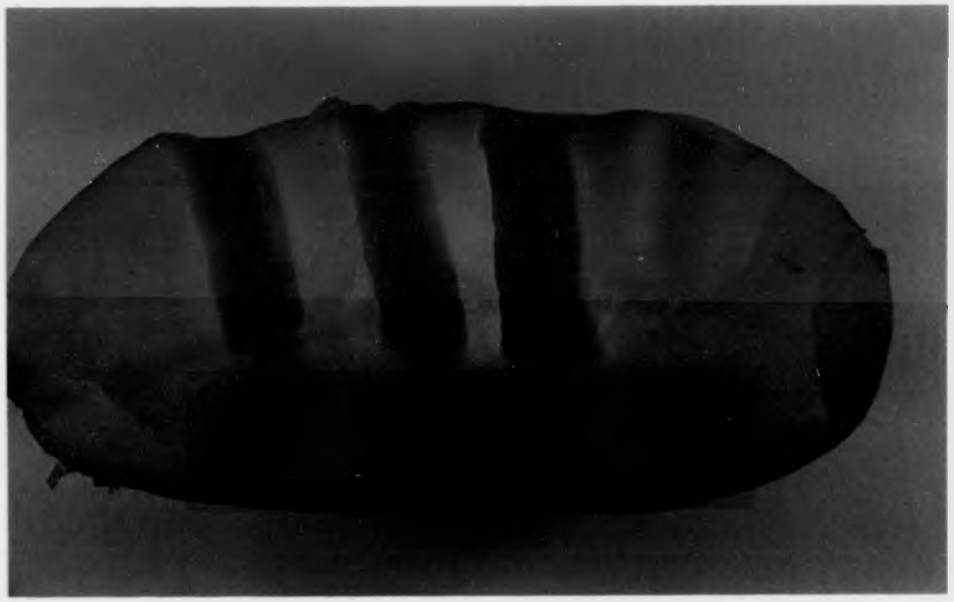


Figure 5.6. An example of a potato used in virulence assays.

The photo shows a potato tuber inoculated with the larger inoculum size, and incubated for 2 days. The potato was sliced through the centre of the inoculation sites, and macerated tissue removed.

restored to wild type levels and localisation in both strains in culture. The most likely explanation for the lack of complete complementation of rotting ability is that the lack of antibiotic selection in potato tissue results in significant plasmid loss. This would reduce cellulase activity levels *in planta* compared with those seen in liquid culture, where antibiotic selection is used.

5.6 Investigation of plasmid stability *in planta*

The stability of plasmids pDEE339 and pSFD1 during growth in potato tuber tissue, in the absence of antibiotic selection, was investigated.

Following inoculation of potato tubers and incubation for 48 hours, as described in section 2.13, the resulting macerated tissue was removed and serially diluted. Aliquots were spread onto LB plates in the absence of selection, and following 24 hours incubation the resulting colonies were picked onto LB containing either kanamycin or spectinomycin as appropriate, to assess the rate of survival. GS7000 + pDEE339 exhibited 13% plasmid loss following such treatment, while GS7000 + pSFD1 exhibited 47% plasmid loss.

These results indicate that in the absence of selection, the level of retention of pSFD1 is very low, and that of pDEE339 slightly reduced. This difference is as one might expect given the large size of pSFD1 (at least 30Kb). The results also coincide well with the ability of these strains to cause soft rot, compared with the wild type. In other words,

the lack of complete complementation of the soft rot deficiency of GS7000 is a result of plasmid instability and the concomitant reduction in cellulase production.

5.7 Expression of cellulase in *E. coli*

In contrast to the results in GS7000, *E. coli* TG1 does not exhibit any degradative ability, even when synthesising CelV at a relatively high level. The amount of rotted tissue resulting from inoculation with TG1 either with or without *celV* shows no difference from that resulting from an un-inoculated stab hole. As the graph of cellulase activity assays (figure 5.1) shows, this rotting deficiency is despite the high levels of activity measured in TG1 + pVIC626. In addition, it seems unlikely that this effect is due to the inability to secrete CelV, because as figure 5.1 shows, high level expression of *celV* results in leakage of some cellulase activity into the external milieu.

5.8 Effects of different inocula

Tubers were inoculated with two different concentrations of cells, neat overnight culture (approximately 10^7 cells in the 10ul sample used) and a ten thousand-fold dilution of this (approximately 10^3 cells). Comparison of figures 5.4a and b indicates that the general effects observed were the same regardless of the inoculum used. In both cases the role of cellulase in maceration is clear in that GS7000 is seriously deficent, but well complemented back to SCRI193 levels by both plasmids; while *E. coli* fails to cause

significant maceration. Interestingly, inoculation with the lower concentration of cells, although initially causing very little rot, by day 4 of the experiment actually causes a greater level of maceration than the larger inoculum.

5.9 Discussion

The results presented here indicate that the cellulase deficient phenotype of the chemically induced mutant, GS7000, results from a mutation within the *celV* locus. Western analysis provides no evidence for synthesis of a wrongly folded form that might clog the secretory apparatus, and since low copy expression of *celV* results in faithful imitation of wild type conditions, and complementation is only effected by *celV*, it is unlikely that GS7000 is mutated at an (as yet uncharacterised) regulatory locus. This enables the use of GS7000 as a clean background in which to study secretion, using expression of *celV* from pDEE339.

Regarding the phytopathological role of cellulase, GS7000 has a significantly reduced ability to macerate potato tuber tissue compared with the wild type SCRI193. This establishes that cellulase must be an important determinant of the ability of Ecc to macerate tuber tissue.

In contrast, there is no evidence for even the slightest ability of *E. coli* to macerate potato tubers when expressing *celV* at a high level. These results are in contrast to those seen for the expression of pectate lyases in *E. coli* already discussed. The obvious conclusion, therefore, is

that although unable to independently macerate host tissue, cellulase seems important nevertheless in the collaborative action of the range of secreted degradative enzymes.

The fact that the lower inoculum eventually overtakes the other in terms of the level of maceration caused is interesting. Initially, it was decided to carry out the experiment with two different concentrations of cells as it was feared that the larger concentration might over-compromise the host in a way not seen in the wild situation, but the results suggest that this might not be the case.

Regarding the reason for this distinction in effects, one might speculate that it is the result of competitive effects. Presumably, the artificial medium LB represents a richer nutrient source than the potato, at least in terms of the ease with which components can be assimilated. The most highly concentrated inoculum contains cells in stationary phase, in that they have reached the level at which the lack of nutrients remaining in the broth becomes a limiting factor. Those which have been diluted, on the other hand, have an excess of rich medium on which to grow. The high cell density of the neat inoculum will mean that initial maceration levels will be high, while those for the diluted inoculum will be low. However, the rate of increase may well be higher, so that once the population becomes established the level of maceration would be more signficant.

A useful additional control in this investigation would have been an additional series of inocula where the overnight

culture was again used neat, but the cells were first re-suspended in fresh LB. This would eliminate the effects of the extracellular enzymes which had already accumulated in the external milieu. One could otherwise envisage a situation whereby the initial levels of enzymes are responsible for virtually all of the rot seen, while cell population levels remain much as they were at the time of inoculation. Indeed, it might also be possible that the high level of initial rot resulting could initiate a defensive response by the plant. This might result in an effect essentially like that of the hypersensitive response observed when a plant is infected by an incompatible pathogen, with the result that localised high-level necrosis protects the plant as a whole from infection (e.g Lindgren *et al.*, 1986). In other words, high level initial degradative enzyme levels could ultimately be a disadvantage to further ingression.

It appears, then, that there exists a synergistic relationship between the various classes of exoenzyme, which is sensible when one considers the compexity of the plant tissue being attacked. A given pathogen may have the ability to degrade a particular host tissue component with great efficiency, but in the absence of the ability to degrade other components, a scaffold of pathogen-resistant structures would remain, inhibiting further pathogenic invasion. Given the abundance and structural resilience of cellulose, it is clear why secretion of an arsenal of pectolytic enzymes is inadequate if a cellulose skeleton remains to stifle further invasion.

CHAPTER 6

EFFECTS OF OVEREXPRESSION OF CELV;

ESTABLISHING A LOW COPY EXPRESSION SYSTEM

6.1 Introduction

Expression of Pel or Cel in Ecc on a plasmid even of moderate copy number, such as pBR322, results in overloading of the secretory apparatus such that these enzymes are not efficiently secreted (Py *et al.*, 1991a). This obviously presents a problem when studying secretion because the wild type system under study will already be partially deficient, making it difficult to identify true secretion mutants. The solution to this problem was cloning of *celV* into a very low copy number vector, thereby retaining the manipulative advantages of plasmid-based expression, but at the same time constructing an experimental system which closely imitates the normal situation of chromosomal expression.

6.2 Cloning into a low copy vector

A 2kb *Sph*1/*Eco*R1 fragment (appendix 1) was cloned into pLG339 (Stoker *et al.*, 1982) cut with the same enzymes (figure 6.1) to give the 7.4kb plasmid pDEE339. This has an SC101 origin of replication, so that the predicted copy number is approximately 6 per chromosome.

6.3 Verification that the copy number is low enough

pDEE339 was expressed in GS7000, a cellulase minus derivative of the wild type SCRI193, constructed by EMS mutagenesis, which was described in chapter 5. Figure 6.2 shows the cellular localisation of cellulase activity in GS7000 + pDEE339, compared to that of SCRI193. As the data show, GS7000 + pDEE339 exhibits slightly higher total

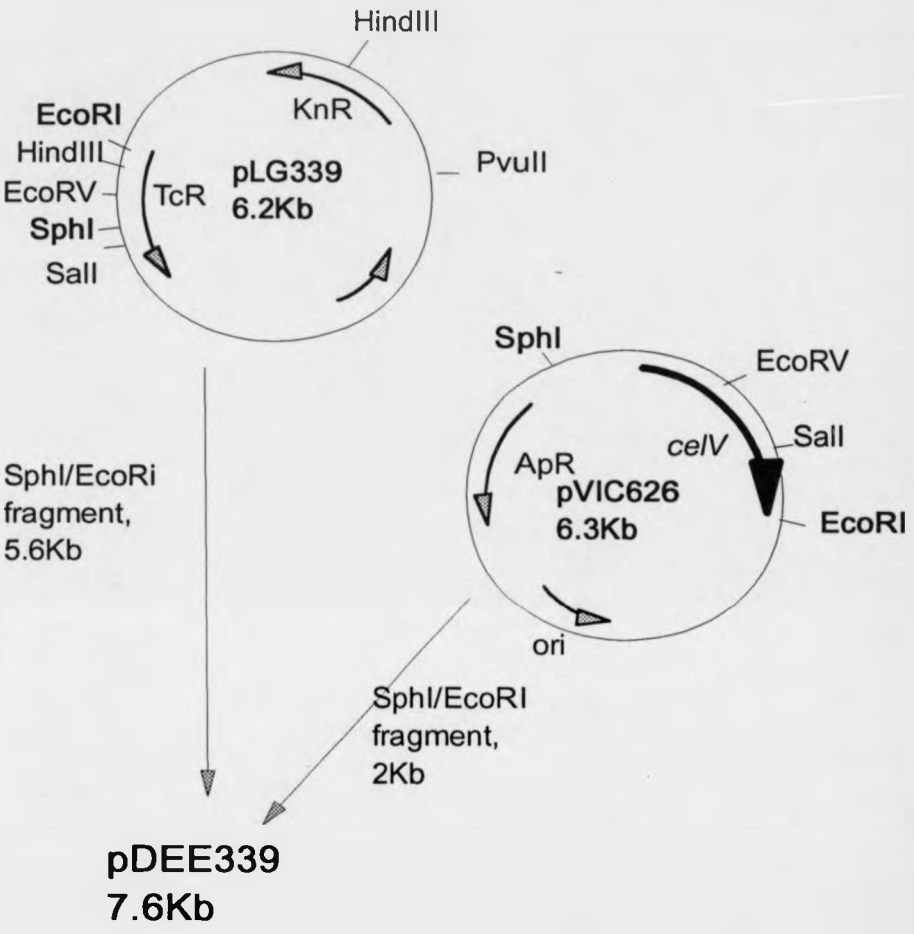


Figure 6.1. Cloning of *celV* into pLG339.

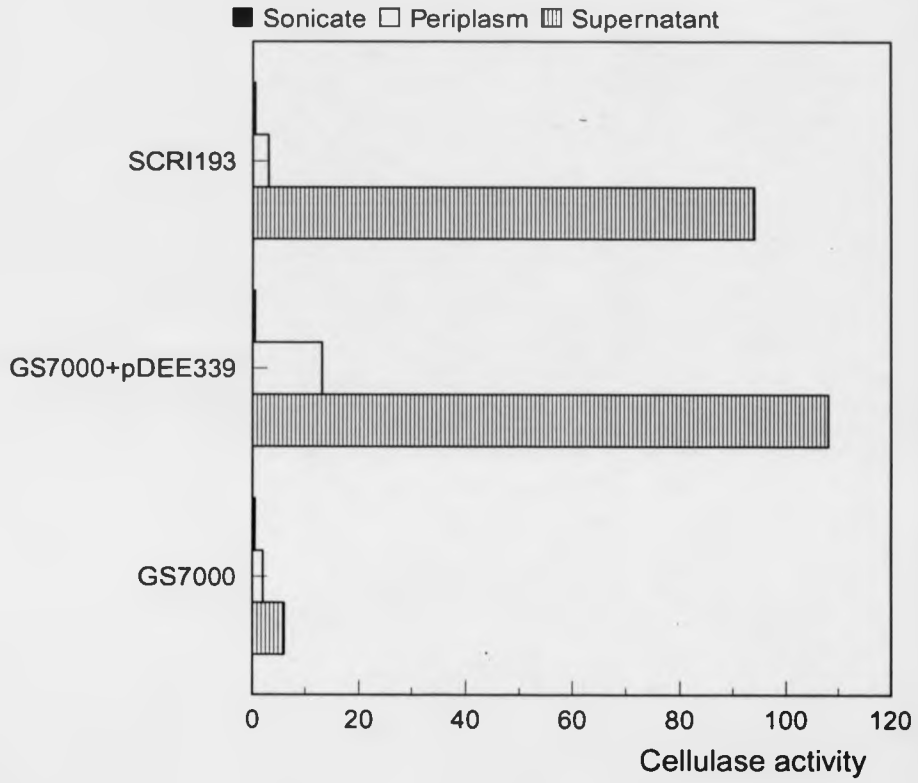


Figure 6.2. Localisation of cellulase activity in GS7000 + pDEE339.

Cell fractions of GS7000 + pDEE339 are compared with GS7000 and SCRI193. Activity is expressed as percentage SCRI193 total activity, based on a standard curve constructed from serial dilutions of SCRI193 whole culture sonicate. Fractionation control: for each strain, at least 93% *B* galactosidase activity was located in the cytoplasmic fraction.

activity, but the majority (at least 85% of total activity, compared with 95% in SCRI193) nevertheless continues to be secreted normally. It appears, judging from the level of cellulase activity, that the copy number of pDEE339 expressed in *Erwinia* may be less than 6.

6.4 Further investigation of overexpression of CelV

The fact that the overexpression of *celV* from a plasmid even of moderate copy number results in jamming of the secretory apparatus, and therefore accumulation of CelV in the periplasm raises several main questions, the answers to which could provide important clues about the nature of secretion in the normal situation.

The first point to make is that, as, for example, Py *et al.*, (1991a), working on *Erwinia chrysanthemi*, and Pugsley *et al.*, (1989), working on pullulanase secretion, have shown, overexpression of proteins secreted by other Out-type apparatuses does not result in a similar overloading effect, which suggests a significant distinction between the mechanisms of secretion.

Secondly, there is the question as to whether overexpression of one of the proteins secreted by the Out apparatus interferes with the secretion of other enzymes which follow the same pathway, or whether these continue to be secreted efficiently. This could provide clues about the pathways followed: whether cellulase and pectate lyases are secreted by precisely the same mechanism or whether the initial

stages involve different pathways which then converge to a common translocation mechanism.

The third consideration is that accumulation in the periplasm as a result of overexpression could identify a rate-limiting step, perhaps as a result of titration of a particular component of the secretory apparatus, so that further investigations could lead to the identification of such a component.

6.5 The effect of overexpression of cellulase on secretion of pectate lyases

An important question regarding the mechanism of secretion is whether overexpression of cellulase also results in the accumulation of pectate lyases, or whether these are still secreted as normal. The answer to this question could provide information about the rate limiting step in the process, and whether cellulase and pectate lyase follow precisely the same pathway.

GS7000 was electroporated (section 2.5.2) with pUCV-1, pUC19 containing *celV* cloned into the multiple cloning site (figure 6.3). A resulting transformant was grown and then fractionated into supernatant, periplasm and cytoplasm fractions as described in 2.2.4, then the fractions assayed for cellulase and pectate lyase activity (sections 2.2.5 and 2.2.6). The results are shown in figures 6.4 and 6.5.

As the graphs show, overexpression of *celV* on a pUC vector

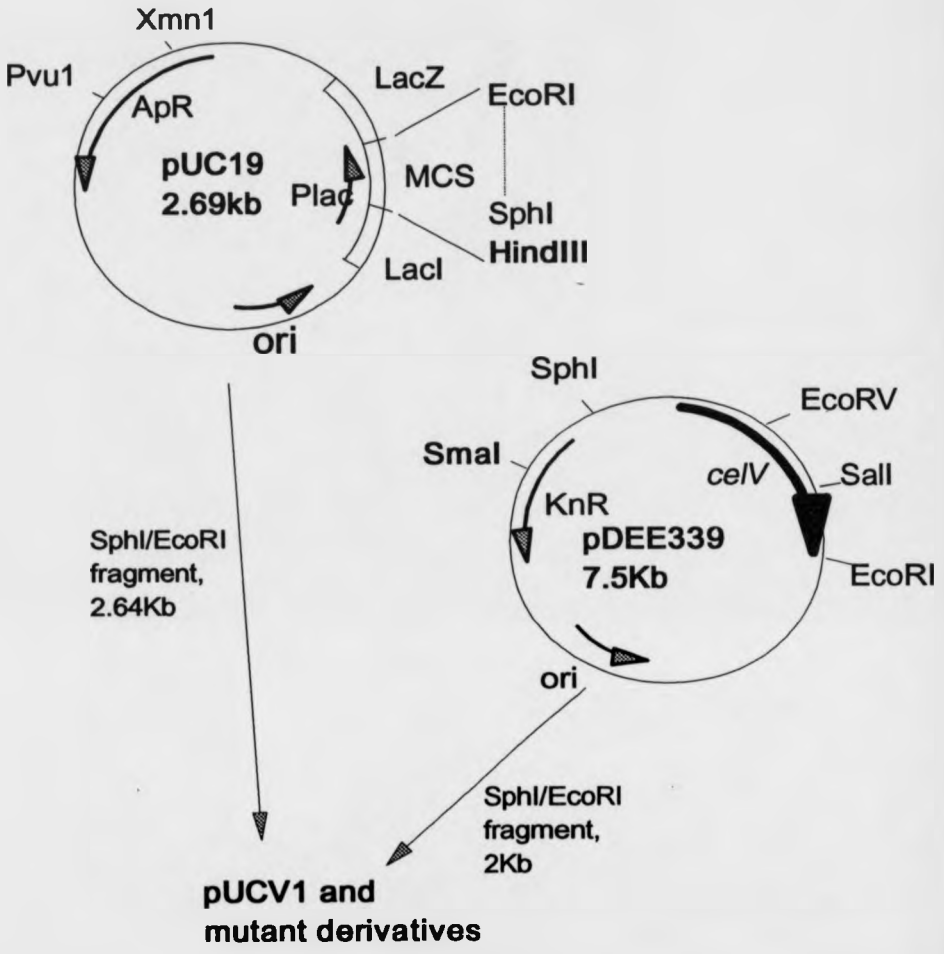


Figure 6.3. Cloning of *celV* into pUC19.

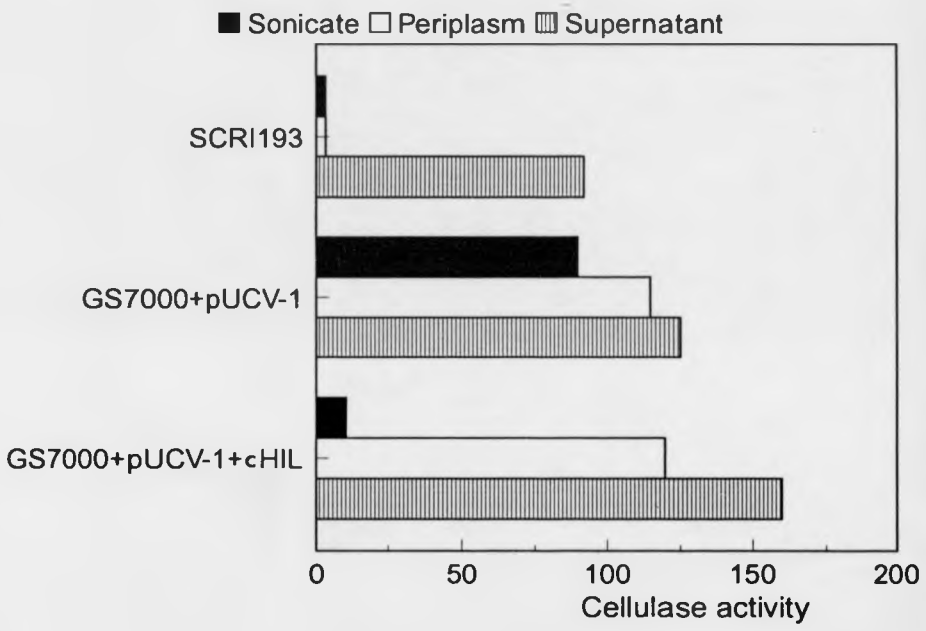


Figure 6.4. Localisation of cellulase activity in GS7000 + pUCV-1.

Cell fractions from this strain are compared with those from SCRI193 and GS7000 + pUCV-1 + cHIL251-3. Activity is expressed as percentage SCRI193 total activity, based on a standard curve drawn from serial dilutions of whole culture sonicate of SCRI193. Fractionation control: for each strain, at least 92% *B* galactosidase activity was located in the cytoplasm, and, where relevant, at least 90% *B* lactamase activity was located in the periplasm.

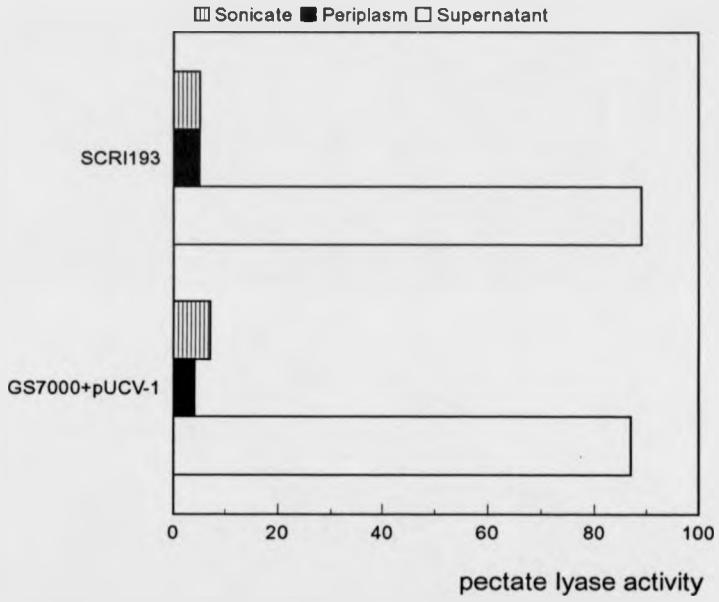


Figure 6.5. Localisation of pectate lyase activity in GS7000 + pUCV-1.

The graph shows pectate lyase activity of cell fractions from GS7000 + pUCV-1 and SCRI193. Activity is expressed as percentage SCRI193 total activity. Fractionation control: see figure 6.4.

results in considerable accumulation of cellulase activity, not only in the periplasm but also in the cytoplasm fraction, while pectate lyase continues to be efficiently secreted. This suggests that the rate limiting step in cellulase secretion is at a stage when cellulase and pectate lyase secretion follow distinct pathways. This could mean that cellulase and pectate lyase are recognised by different receptors in the Out apparatus, or that periplasmic folding is aided by different chaperones, or indeed that folding of pectate lyase does not require a chaperone while that of cellulase does. One other possibility is that the high level of accumulation of cellulase results in the formation of aggregates, rendering the cellulase molecules involved unsecretable. This last scenario could also explain the apparently high level of accumulation in the cytoplasm; this could be an artefact, due to the inability of cellulase aggregates to dissociate efficently during spheroplasting.

6.6 Is cellulase accumulated in the periplasm still in a secretable state?

There are a number of possible explanations for the periplasmic accumulation of cellulase. The rate limiting step could be the release or emergence of cellulase from the Sec pathway in the inner membrane, the folding of cellulase in the periplasm into a secretion-competent form, perhaps with the aid of a periplasmic chaperone, the recognition of cellulase by a certain Out-specific receptor, or its translocation across the outer membrane by the Out complex.

Investigation as to whether the cellulase accumulated in the periplasm is still competent for secretion will help to establish which of these possibilities is most likely to be the case.

GS7000 + pUCV-1 was grown in LB and appropriate selection to A_{600}=1.2, and a sample taken for fractionation. The culture was then split into two and each centrifuged and the supernatant discarded. Each was resuspended in the original volume of LB, Ap, then samples taken for fractionation. To one culture, chloramphenicol was added to a final concentration of 50ug/ml, with the purpose of arresting synthesis. Growth, shaking at 30°C was then continued, samples being removed at 2.5, 5, 10, 20, 40 and 80 minutes from the time when chloramphenicol was added. For all of the samples taken, optical density was measured, cells were fractionated into supernatant, periplasm and cytoplasm fractions (section 2.2.4), and supernatant samples concentrated 10 times using TCA precipitation (section 2.11.4). Samples were then analysed by Western blotting (sections 2.1.4 - 2.1.6) using anti-CelV polyclonals; and using cellulase and pectate lyase activity assays (sections 2.2.5 and 2.2.6). Figure 6.6 shows the results of enzyme activity assays and optical densities for comparison; figure 6.7 shows the results of Western analysis.

The plot of optical density in figure 6.6 confirms that the addition of chloramphenicol produces a significant effect on growth rate, due to an arrest of synthesis.

Figure 6.6. Is cellulase accumulated in the periplasm still in a secretable state? Cellulase assays.

The graphs show, from top to bottom, optical density of culture; cellulase activity in supernatant; cellulase activity in periplasm; cellulase activity in sonicate (i.e. cytoplasm).

The experiment is described in detail in the text. The T = 0 sample is the initial culture; T = 0' is the sample following washing of the cell pellet and resuspension in fresh LB (just before chloramphenicol was added to one culture); T = 2.5 is the sample taken 2.5 minutes following the addition of chloramphenicol and continuation of incubation at 30°C, with shaking; T = 5 is the sample taken 5 minutes after the addition of chloramphenicol, and so on.

Activity is expressed as percentage SCRI193 total activity, using a standard curve constructed from serial dilutions of whole culture sonicate, using a culture grown to A_{600} = 1.3.

Fractionation control: at least 91% *B* galactosidase activity is in the sonicate, while at least 92% *B* lactamase activity is located in the periplasm.

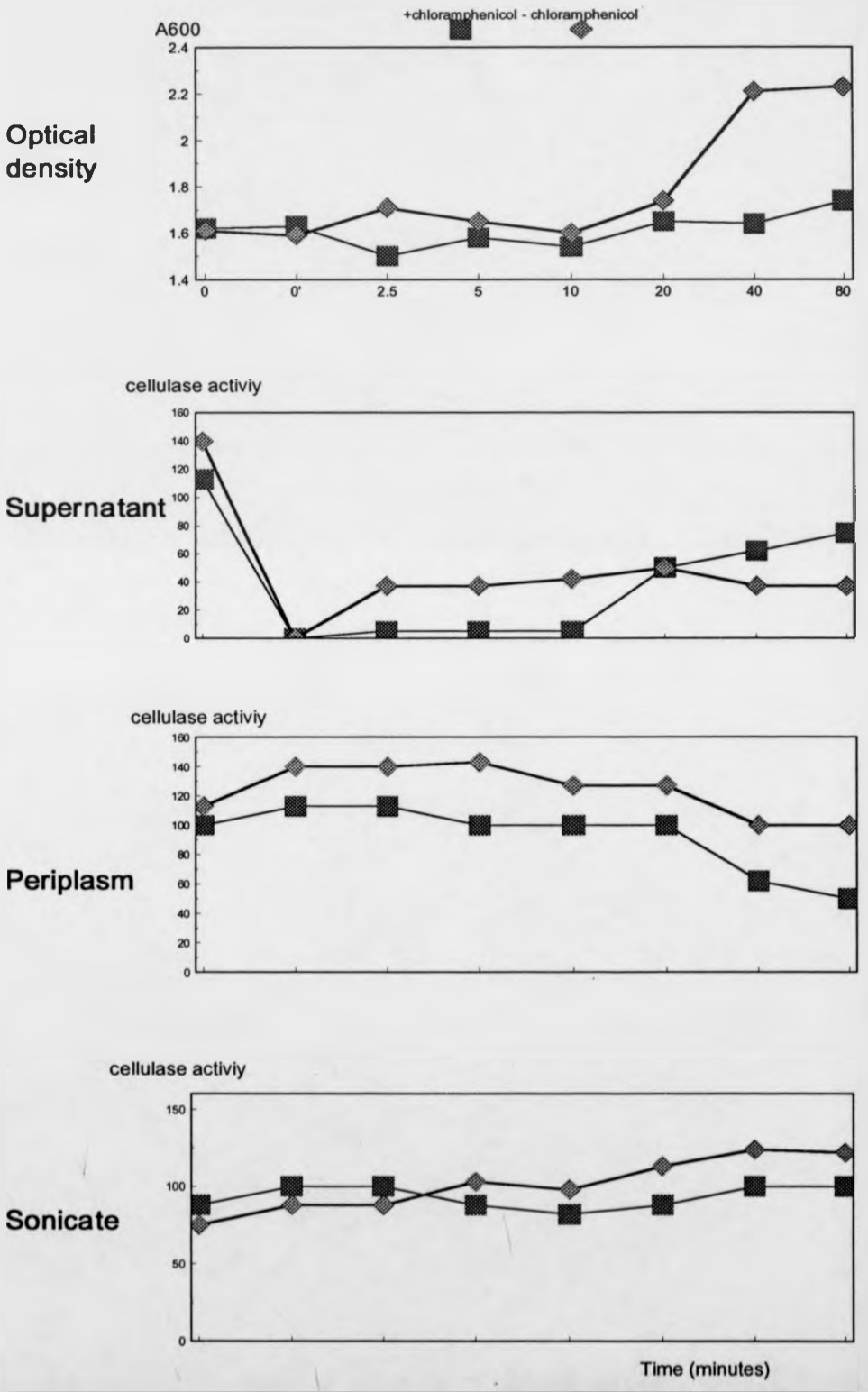
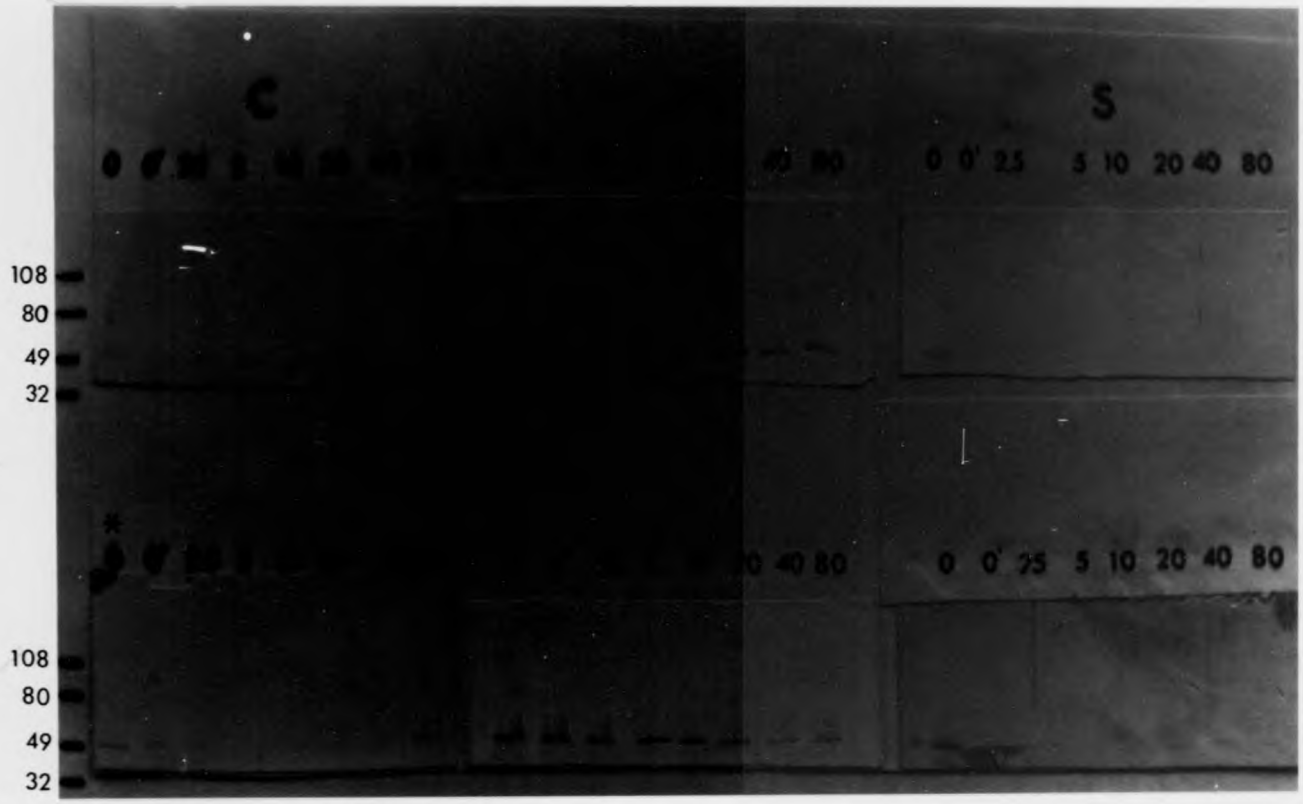

+chloramphenicol - chloramphenicol
A600
Optical density
2.4
2.2
2
1.8
1.6
1.4
0
0'
2.5
5
10
20
40
80
cellulase activiy
Supernatant
160
140
120
100
80
60
40
20
0
cellulase activiy
Periplasm
160
140
120
100
80
60
40
20
0
cellulase activiy
Sonicate
150
100
50
0
Time (minutes)

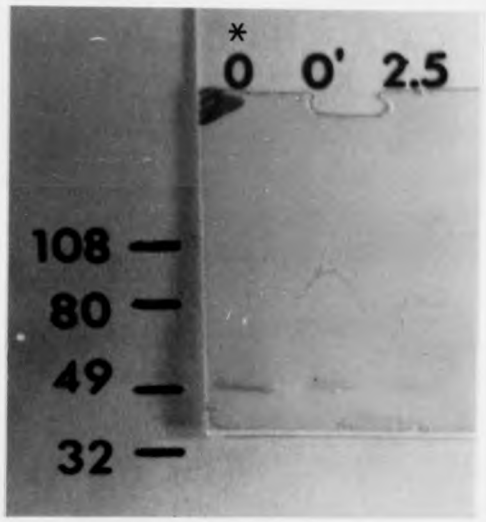
Figure 6.7. Is cellulase accumulated in the periplasm still in a secretable state? Western analysis.

(A) (opposite) shows Western analysis of the samples described in figure 6.6, using Anti-CelV polyclonal antibodies. * The sonicate fraction from T = 0, without chloramphenicol, has been replaced by the periplasmic fraction from the same sample, to enable comparison of the molecular weight of cytoplasmic and periplasmic forms. Molecular weight standards are given on the right, in KDa.

(B) (following page) shows part of the gel of sonicate fractions from the experiment without chloramphenicol in greater detail. * again denotes replacement of the T=0 sonicate sample with the corresponding periplasmic fraction.


C
S
0 0' 2.5 5 10 20 40 80
108
80
49
32
+Cm
*
0 40 80
0 0' 2.5 5 10 20 40 80
108
80
49
32
-Cm

B



The activity seen in the supernatant is initially very high, and drops to zero at T=0', when cells are pelleted and resuspended in fresh medium. Where chloramphenicol is not added to the culture, cellulase levels in the supernatant gradually build up again and indeed are clearly visible by Western analysis after about 10 minutes. Secretion is not, however, very efficient, although this could simply be a reflection of the clogging up of the system as a result of high level accumulation. In the presence of chloramphenicol, activity is slower to appear in the supernatant, but does nevertheless clearly appear, although only barely detectable by Western analysis at the final, 80 minute time-point. Meanwhile, the level of activity visible in the periplasm drops considerably in parallel with the supernatant increase. This indicates that cellulase which had accumulated in the periplasmic fraction is still capable of being secreted, although this conclusion would be confirmed by a pulse-chase experiment, since this would allow us to follow the path taken by a subpopulation of CelV molecules.

Concerning events in the cytoplasm, the experiment in which chloramphenicol was added shows very little change in cellulase activity in the sonicate sample over the course of the experiment. This suggests that cellulase accumulated in the sonicate fraction is not secreted, and perhaps that it no longer able to be secreted. As already discussed, the level of activity seen in this fraction may be an artefact of the spheroplasting method used. A large proportion of the cellulase detected could be membrane associated (perhaps because release from the Sec apparatus is the rate-limiting

step) or could form aggregates which do not fractionate properly.

The situation is clarified by consulting the Western analysis of sonicate fractions, in figure 6.7. The T=0 sample for the experiment without chloramphenicol (which is identical to the T=0 sample of the parallel experiment) is replaced by the periplasmic fraction of the same sample. This provides a molecular weight control, allowing direct comparison of the size of periplasmic and cytoplasmic forms of the enzyme. The gel shows that the periplasmic form runs slightly faster, as one would expect, given the cleavage of the signal sequence. In addition, only one species, the unprocessed one, is visible in the sonicate fraction. This indicates that the fractionation procedure used is successful, from the point of view that the uncleaved species localises exclusively to the sonicate, while the cleaved species is not detectable in this fraction.

Comparison of periplasm and sonicate samples side by side on the same gel also indicates that the periplasmic band is more intense, although, as figure 6.6 shows, there is very little difference in cellulase activity. This raises the possibility that there exists a catalytically active form which is not recognised by the antibodies. There are two possible explanations. The first is that cellulase which fails to associate with a SecB homologue in the cytoplasm is proteolytically cleaved into the two domains, the catalytic domain remaining active but not being recognised by the antibodies used. The second possibility is that there exists

a periplasmic form that as a result of failure to associate with a periplasmic chaperone forms secretion-incompetent aggregates that are not recognised by the antibodies and fractionate artefactually with the sonicate. This is reminiscent of investigations of the role of the *E. coli* periplasmic chaperone, PapD, (Kuehn *et al.*, 1991) which directs pilin subunits to outer membrane assembly sites. In the absence of PapD, pilin subunits form non-productive aggregates. This second possibility could only explain the observations if such a form failed to be recognised by the antibodies, but retained catalytic activity.

Concerning the control experiment in the absence of chloramphenicol, cellulase levels continue to rise, as expected, with a particularly high level of accumulation in the sonicate fraction.

6.7 Complementation of the overloaded secretion apparatus

It was important to determine whether the overloading effect resulting from overexpression of CelV can be compensated for by overexpression of Out proteins. If the rate limiting step was some sort of periplasmic chaperone, such an approach would be useful in trying to identify this.

GS7000 + pUCV-1 was electroporated (section 2.5.2) with cHIL251-3, a pSF6-based plasmid carrying *outC-O* and flanking sequences, and transformants selected in the usual way, on LB, ampicillin, spectinomycin plates. The resulting strain was very slow growing: a 5ml culture, loop inoculated in LB,

reached an optical density of A_{600} = 1.4 after 23 hours, compared with 16 hours for GS7000 or SCRI193, and 19 hours for GS7000 + pUCV-1. Figure 6.8 shows the results of cellulase activity assays (section 2.2.5) carried out on cell fractions (section 2.2.4) GS7000 + pUCV-1 with and without cHIL251-3. The results appear to suggest that high level production of the Out proteins can indeed compensate for the secretion deficiency that results from over-production of CelV. However, it is difficult to draw any reliable conclusions when the growth rates of the two strains are so different, since we have no way of predicting the effects of this. It would therefore be very interesting to investigate this effect further, using smaller subclones of the *out* cluster, constructing smaller plasmids that would exert less of a load on growth of the organism, to try to identify which Out protein(s) are responsible.

6.8 Discussion

The results presented here indicate that in the low copy expression system established, expression of CelV is at an adequately low level to allow relatively efficient secretion. GS7000 + pDEE339 can therefore be used as a model system which closely imitates SCRI193 but is genetically amenable from the point of veiw of isolation and characterisation of secretion deficient mutants.

Concerning the investigations of the overloading of the secretory apparatus by overproduction of CelV, the main conclusion is that this is a phenomenon which merits further investigation, because it can provide important clues

regarding the mechanisms of secretion.

The experiment is difficult to interpret because the level of detection of the antibodies and the level of accuracy of the cellulase assays could result in considerable variation due to experimental error. This is particularly true of the sonicate fraction, because it could interfere in the ability to detect secretion of a portion of this component. The clear decline in periplasmic activity, combined with the steady maintenance of sonicate activity suggests, however, that this would not be the case.

Although requiring confirmation by a pulse-chase experiment, the results presented suggest that cellulase accumulated in the periplasmic fraction is still in a secretable form, while that in the sonicate fraction is not. The conclusions which can be drawn from this, however, depend on the exact nature of the sonicate form.

As already discussed, one alternative is that the sonicate fraction consists of a cytoplasmic form which is recognised by the anti-CelV antibodies, and a periplasmic form which, as a result of failure to associate with a secretion chaperone, forms aggregations which are not recognised by the antibodies. The portion which accumulates free in the periplasmic space and is still secretion competent would be assumed to be chaperone-associated, while the aggregated remainder is no longer secretion-competent. The apparent ability of overexpression of the Out apparatus to complement the secretion deficiency could imply that one of the Out

proteins is a periplasmic chaperone which maintains the enzyme in a secretion competent state. The alternative is simply that all of the cellulase fractionating with the sonicate is in the cytoplasm, and that a proportion of this has degraded into the constituent domains as a result of sequestering of SecB. However, one would expect such a defect to affect secretion of other proteins. It is clear, then, that further investigations are necessary.

The most useful approaches now would be:-

1) A pulse chase experiment, to radioactively label cellulase produced over a short period of time, then following its progress to confirm that cellulase in the periplasmic fraction is indeed secreted, and to determine whether there is a contingent that remains permanently blocked in the sonicate fraction.

2) To carry out further localisation experiments, to identify the nature of the CelV blocked in the sonicate sample.

3) To extend complementation studies, to identify Out protein(s) which can compensate for the effect, and therefore to implicate them perhaps as periplasmic chaperones, or at least as being part of the rate limiting step. A major problem with this approach is that the stoichiometry of formation of the Out complex can mean that expression of part of the gene cluster can interfere with complex formation. The best approach, therefore, would be to express Out proteins individually.

CHAPTER 7

ISOLATION OF SECRETION MUTANTS

7.1 Mutagenesis strategy

The strategy chosen for the isolation of secretion deficient mutants was localised mutagenesis of the cellulase gene *celV*. The aim was to isolate point mutants deficient in secretion, with a view to identifying particular residues which are important to secretion, and thereby try to identify some sort of motif required for recognition of CelV by the Out apparatus.

The aim was to isolate mutants that were catalytically active but deficient in secretion, in other words, mutants that showed close to wild type activity, but with this activity localised partially or completely in the periplasmic fraction. The reasons for taking this approach were both theoretical and practical. Retention of catalytic activity was used as an indication of reasonable conformational integrity of the protein. Many situations can be envisaged in which a point mutation can result in the destruction of both catalytic activity and secretability, and these might include nonsense mutations resulting in truncation of the protein, or gross conformational changes. The latter are particularly important considering the growing body of evidence for the requirement of at least a certain degree of folding in the periplasm, as discussed in chapter 1.

In addition, from the point of view of the initial screening on carboxymethyl cellulose plates, point mutations which result in down regulation; or in the case of hydroxylamine

mutagenesis (carried out directly on pDEE339), mutations interfering with plasmid replication, would also exhibit the same phenotype, since the lack of cellulolytic activity prevents localisation of the enzyme.

A further consideration is that, from a practical point of view, mutagenesis rates must be kept relatively low in order to reduce the likelihood of double mutations, since these would complicate further investigations. This means that the number of transformants screened must be high, and so the only practical method of screening for secretion mutants is by picking colonies onto carboxymethyl cellulose plates. Therefore, mutants which are deficient in catalytic activity represent unwanted background interference.

7.2 PCR mutagenesis

The strategy used in mutagenesis by polymerase chain reaction (PCR) was based on the data presented by Leung *et al.* (1989). The idea was that the fidelity of *Thermus aquaticus* polymerase (Taq) can be reduced either by altering the ratio of dNTPs so that one is present at a reduced concentration compared with the other three, or by the addition of manganese. By playing with these conditions it should be possible to set the rate of mis-substitution by Taq polymerase to give maximal mutagenesis while still ensuring that the rate of mutagenesis is not so high as to make the occurence of double mutants likely.

For the purpose of PCR amplification a 2kb *Sph1/EcoR1*

fragment containing *celV* was cloned into pBluescropt SK- (Stratagene), cut with *Sal*1, *EcoR*1 in the multiple cloning site. For this purpose, restriction sites were blunted (section 2.3.8), such that the new construct retained the *Sal*1 site but lost the *Sph*1 site (figure 7.1). The multiple cloning site is flanked by T7 and T3 promoters, allowing the use of commercial primers to these (Stratagene; table 2.4) to be used in the PCR reaction.

The PCR conditions were as described in section 2.12, having optimised concentrations of $MgCl_2$ and dNTPs, and having adjusted the annealing temperature to give maximum amplification, but maintaining a discrete band of amplified product.

As table 7.1 shows, post-mutagenesis screening of the product achieved from standard PCR conditions (although these conditions were perhaps not highly efficent) resulted in a high level of mutagenesis, so that plans to add manganese or alter the dNTP ratios were abandoned.

7.3 Hydroxylamine mutagenesis

Hydroxylamine mutagenesis was carried out directly on pDEE339 as described in section 2.8.1. Previous studies (I. Bortoli-German, pers. com.) indicated that 36 hours exposure to hydroxylamine resulted in approximately 0.7% CMC^- when EGZ of Ech was expressed in TG1. Therefore 34 hours and 38 hours were the time periods chosen for exposure, and the mutant frequencies resulting from subsequent screening in *E.*

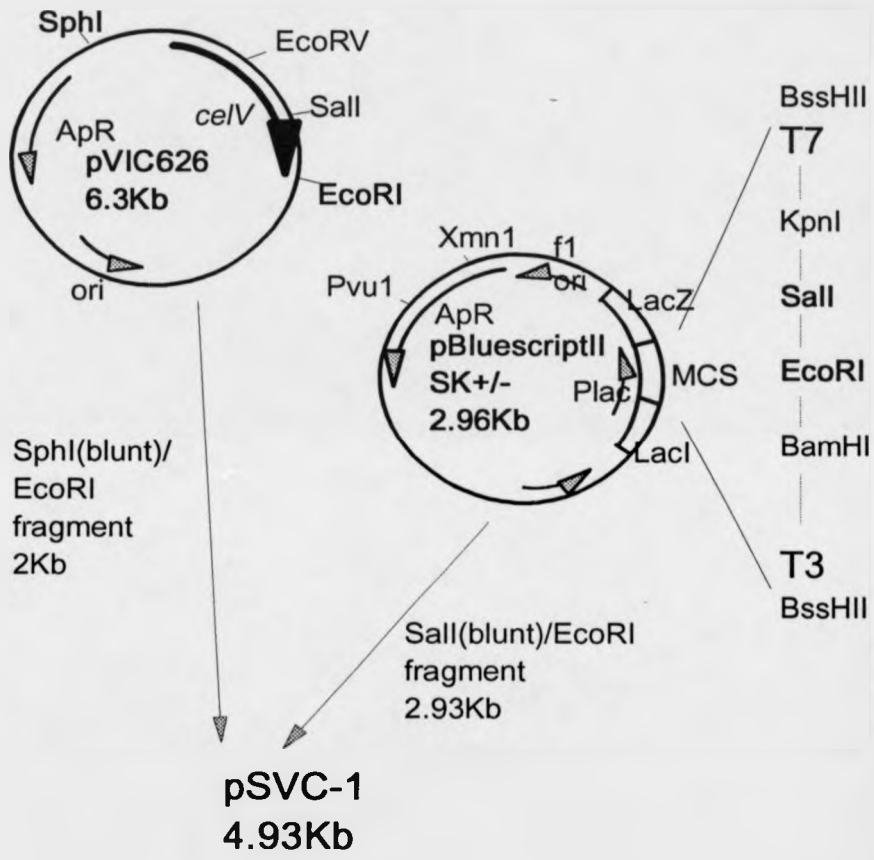


Figure 7.1 Cloning of *celV* into pBluescript.

Mutagenesis Method	Relevant Information	Percentage Cel⁻ In TG1	In GS7000
PCR	*Sph*1/*Eco*R5 fragment	2.2	1.7
	*Eco*R5/*Eco*R1 fragment	1.6	1.2
Hydroxylamine	34 hours exposure	0.48	0.46
	38 hours exposure	0.91	0.85

Table 7.1 Levels of mutagenesis observed for the methods used in this study.

Results were determined by screening colonies on CMC plates. The table shows, from left to right, the mutagenesis method used; relevant information: the subcloned fragment under analysis for PCR mutagenesis or the time of exposure to hydroxylamine; and the percentage of cellulase minus transformants, when potentially mutant derivatives of pDEE339 were screened in *E. coli* TG1 and Ecc GS7000.

coli and *Ecc* are given in table 7.1.

7.4 Post-mutagenesis screening

As figure 7.2 illustrates, both approaches to mutagenesis entailed a two stage screening process, firstly in *E. coli* and then in *Ecc*. The reasoning behind this was the prediction that a CMC$^+$ phenotype when expressed in *E. coli* would indicate that conformationally the protein was relatively normal. Then, when expressed in *Ecc* any isolate which now exhibited a reduced halo would presumably have a good chance of being deficient in secretion, with activity accumulated in the periplasm.

On the other hand, anything that was CMC$^-$ in *E. coli* would then be CMC reduced or minus in *Ecc*, and this could be for any number of reasons; catalyic, regulatory or conformational.

The mutagenised pDEE339 derivatives from both mutagenesis methods were introduced into *E. coli* by PEG transformation (section 2.5.1). Transformants were screened on CMC assay plates and those exhibiting a CMC$^+$ phenotype were used to inoculate a heterogeneous culture. A minipreparation (section 2.3.2) of plasmid DNA from the heterogeneous culture was introduced into Ecc GS7000 by electroporation (section 2.5.2). The resulting transformants were screened on CMC assay plates, and those showing reduced halos were investigated further.

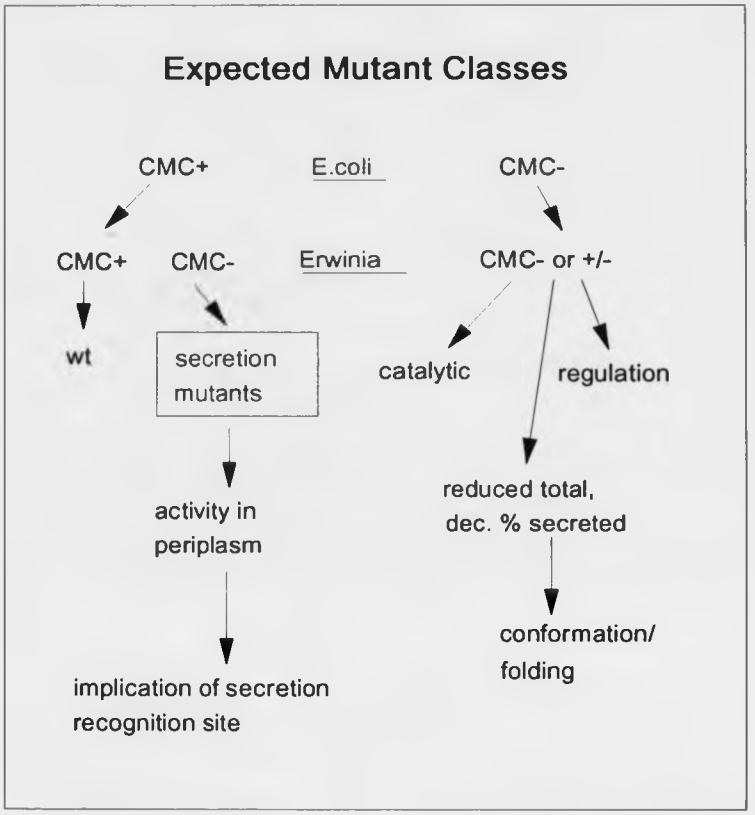


Figure 7.2 Mutant classes predicted to result from the mutagenesis strategy used.

Fig 7.3 shows examples of assay plates from the screening procedure. It is worth pointing out that the concept of CMC$^+$ is very different in the two genera. In *E. coli* cellulase, of course, is not secreted. However, the presence of activity accumulated in the periplasm is still detectable, and still easily distinguished from lack of activity; while in *Ecc* the presence of a large halo indicates secretion of a high level of activity, while secretion deficient and catalytically deficient clones exhibit reduced haloes.

For transformants exhibiting reduced halo size, cultures were grown in LB to A_{600}=1.6-2 (early-mid stationary phase), and fractionated (section 2.2.4) to give supernatant, periplasm and sonicate (i.e. cytoplasm) samples, which were assayed for cellulase activity (section 2.2.5.).

7.5 Mutants isolated

Screening of approximately 1800 transformants from PCR mutagenesis, and 6000 from hydroxylamine mutagenesis resulted in the isolation of eleven mutant derivatives of pDEE339 (one from PCR, ten from hydroxylamine) which expressed cellulases exhibiting total levels of activity which were close to wild type, but with the majority of activity in the periplasmic fraction, rather than being secreted into the supernatant.

Figure 7.4 shows the results of cellulase activity assays for these mutants compared with the wild type, GS7000 + pDEE339. Figure 7.5 shows examples of the CMC well assays

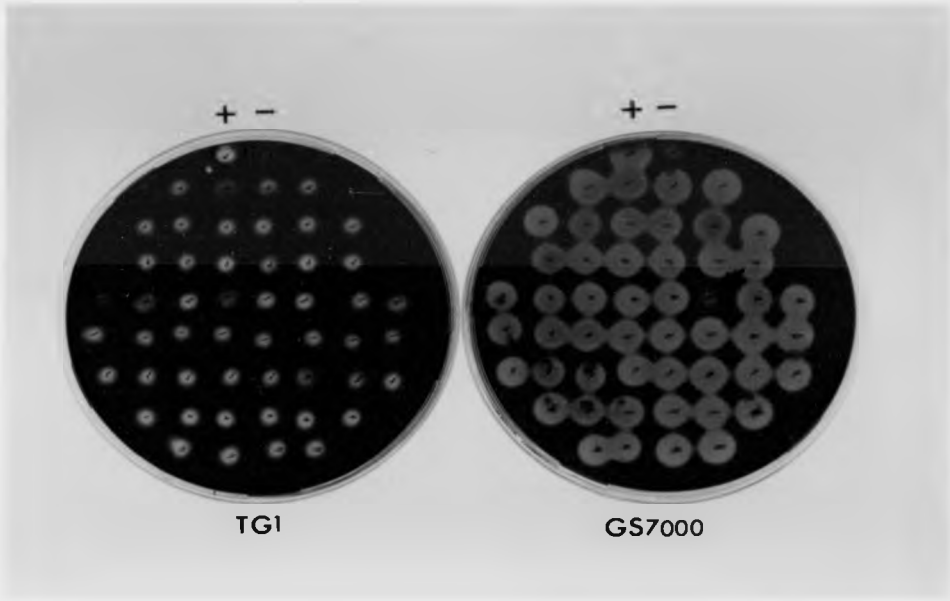


Figure 7.3 Examples of the assay plates used in screening for mutants.

Strains are shown below the plates. The top two positions on each plate, labelled + and −, are controls, SCR1193 and GS7000, respectively. Arrows indicate Cel colonies.

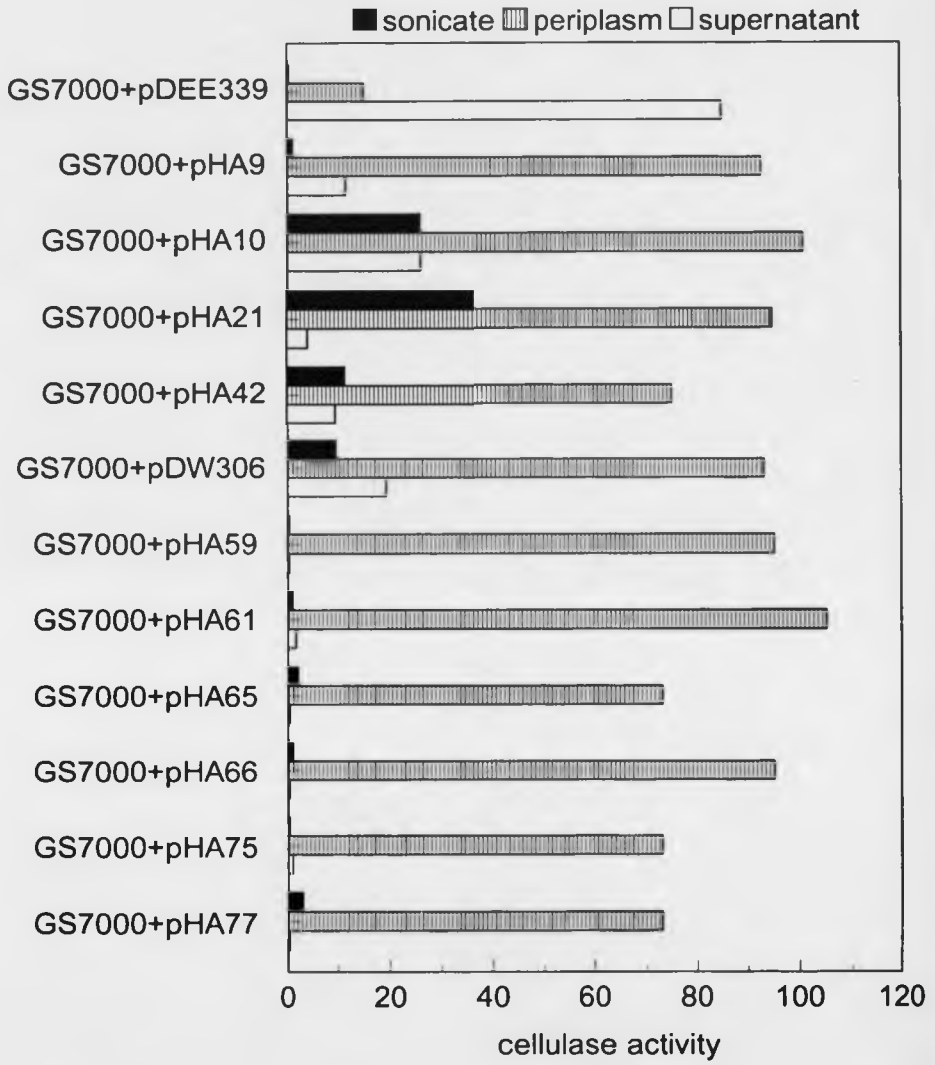


Figure 7.4 Cellulase assays of secretion mutants.

The graph shows cellulase assays of sonicate, periplasm and supernatant fractions from GS7000 + pDEE339, compared with the eleven mutant derivatives isolated. Activity is expressed as percentage SCRI193 total activity, based on a standard curve constructed using a series of dilutions of SCRI193 whole culture sonicate.

Fractionation control: at least 91% *B* galactosidase activity is located in the sonicate.

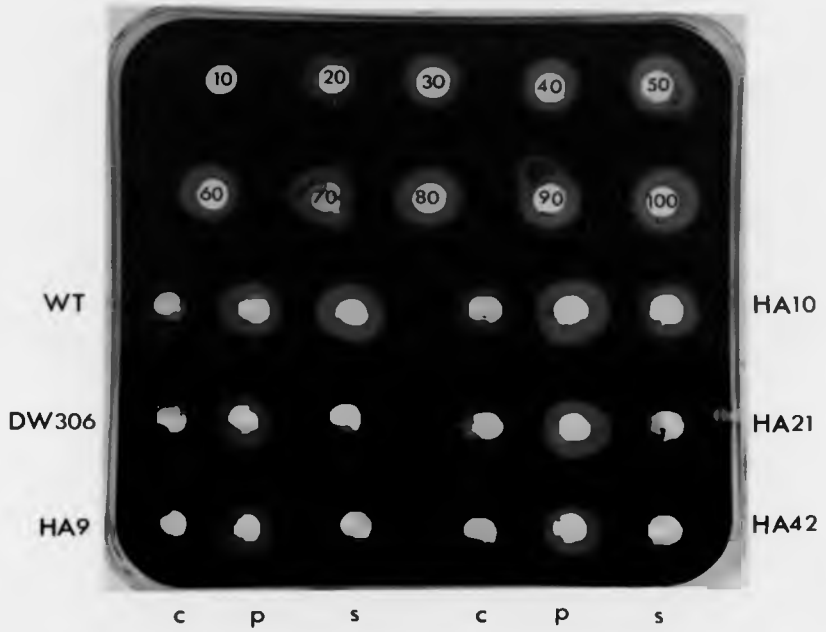


Figure 7.5 CMC well assays of secretion mutants.

An example of one of the CMC well assay plates used to calculate cellulase activity of cell fractions from secretion mutants, as described in figure 7.4. The top two rows show serial dilutions of SCR1193 whole culture sonicate, from 10% to 100%, as marked in the centre of each halo. For the remainder, strains are indicated at the sides (WT represents GS7000 + pDEE339), while cell fractions are indicated at the bottom. S = supernatant; P = periplasm; C = cytoplasm.

which were used in collaboration with the OBR-cellulose assay (section 2.2.5). pDW306 was isolated from PCR mutagenesis of the *EcoRV/EcoRI* fragment, while pHA9 to pHA77 were isolated from hydroxylamine mutagenesis on the whole plasmid.

7.6 Discussion

The strategy chosen for the isolation of mutants which were catalytically wild type but deficient in secretion was successful in that eleven such mutants were isolated. The frequency at which these occurred was relatively low, although this was largely due to the low level of mutagenesis chosen in order to diminish the likelihood of double mutations arising.

The high rate of mutagenesis observed following PCR amplification of *celV* raises important questions regarding the advisability of using Taq polymerase to amplify DNA whose detailed structure, and particularly sequence, is to be investigated. Although the officially stated error rate of Taq is much lower (incorrect substitutions, for example, are estimated to occur at a frequency of 1 in 9000 bases) than that observed in this experiment, the results illustrate that reaction conditions supposed to be standard in fact can result in a significant rate of error, a problem aggravated further as the size of the fragment to be amplified increases. This illustrates the importance of generating several independent isolates of a given amplified fragment if analysis such as sequencing is to be carried out.

CHAPTER 8

CHARACTERISATION OF SECRETION MUTANTS

8.1 Introduction

A potential problem with the mutagenesis strategy used is that success relies on the information for secretion and that for catalytic activity being mutually exclusive, which is a somewhat simplified ideal. This is compounded further by the fact that there is considerable evidence that the secreted protein acquires a periplasmic conformation which is at least partially folded. Secretion is dependent on some degree of conformational integrity. This is illustrated by the dependence on disulphide bond formation which has been demonstrated, for example, in the secretion of pullulanase by *Klebsiella pneumoniae* and of EGZ by *Erwinia chrysanthemi*, as described in the chapter 1. The problem that this presents is that catalytic activity is also to a great extent dependent on conformation.

The dependence of secretion on conformational integrity may be a reflection of the need to retain certain secretion information in a particular orientation or motif, or it may simply be a reflection of the susceptibility of unfolded proteins to periplasmic proteases.

A further complication is that, as Py *et al.* (1991b) showed for EGZ, and V. Cooper (pers. com) demonstrated for CelV, the catalytic and cellulose binding components represent discrete domains which are functionally independent. In addition, treatment with protease *in vitro* (Py *et al.*, 1991b) results in cleavage of the extended linker region, and release of the two domains. Therefore, a mutation which

results in incorrect folding in the periplasm may result in susceptibilty to proteolytic cleavage, resulting in release of a catalytic domain which is fully active but, due to the absence of all the information required for secretion, is not secreted. In view of this it is very dangerous to assume that catalytic activity provides an indication that the protein is normal in all but its secretability.

It is difficult to gain any information regarding the requirements for secretion from mutants that are deficient conformationally since there is no evidence that the residue affected by the mutation is in any way connected with secretion. Therefore, the first step in characterisation of the mutants isolated was to generate more information that will provide an insight into whether they are *bona fide* secretion mutants. The most obvious and simple way of doing this was Western analysis to establish whether a full size, stable and antigenic product could be detected.

In addition, it was important to investigate the effect that the mutant derivatives of CelV had on the secretion of other proteins that are translocated by the Out apparatus, not only heterologous enzymes such as pectate lyases, but also wild type cellulase. The reasoning was that this would provide information as to the stage within the secretion pathway at which the mutant derivative of CelV became blocked, and therefore the nature of the deficiency, and the nature of the interaction with the Out machinery.

Finally, it was important to sequence the mutant derivatives

of *celV* to establish the precise nature of the mutations responsible.

8.2 Western analysis of secretion mutants

Supernatant, periplasmic, and cytoplasmic samples were prepared from the eleven mutant derivatives of GS7000 + pDEE339 (section 2.2.4). Supernatant and cytoplasmic fractions were concentrated ten times by TCA precipitation (section 2.12.4). Samples were boiled in TSTD (section 2.12.4), then subjected to SDS PAGE on Phast gels and Western analysis using purified polyclonal antibodies against CelV (section 2.12.5 and 2.12.6). The results are shown in figure 8.1. The gel shows, as a control, GS7000 + pDEE339, for which a band corresponding to CelV is seen at approximately 50KDa, and is located almost entirely in the supernatant fraction, although a slight band can be seen in the periplasmic fraction.

Regarding the mutant derivatives, only 5 of the 11 exhibit a full size stable product, at least one which is detectable by these antibodies: pHA10, pHA21, pHA61, pHA66 and pHA77. The other 6 do not possess such a product. This suggests that the mutant forms of CelV encoded on pHA10, pHA21, pHA61, pHA66 and pHA77 are bona fide secretion mutants, since they are stable full size proteins, which are catalytically active, and therefore, so far as can be established, are only deficient in secretion. The other six mutants, on the other hand, are more likely to be general, conformationally defective mutants, or truncates, which are

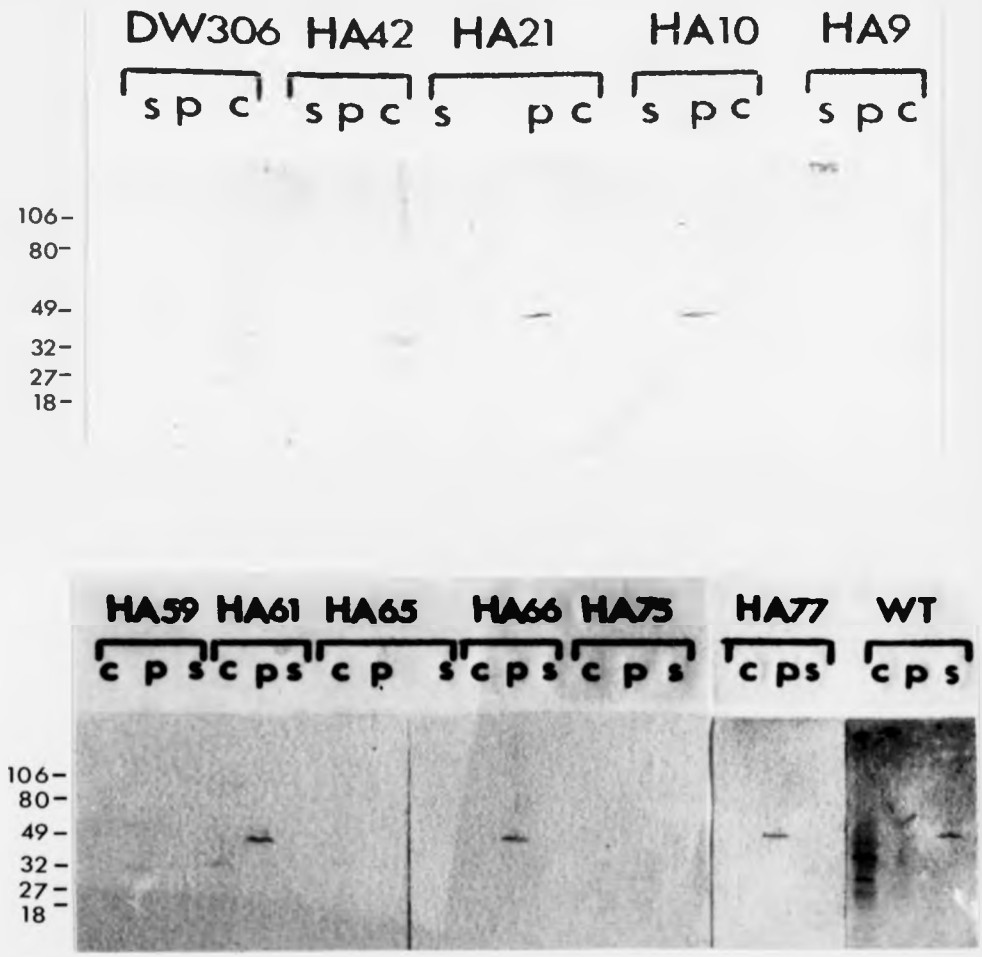


Figure 8.1 Western analysis of secretion mutants, using anti-CelV polyclonals.

Western analysis of the eleven secretion deficient mutants isolated, compared with the wild type, GS7000 + pDEE339, indicated as WT. Strains are shown across the top of gels. S = supernatant; P = periplasm; C = cytoplasm. Positions of molecular weight standards are given on the left, in KDa.

nevertheless wild type for catalytic activity, but about which it will be very difficult to draw any conclusions, since the mutations resulting in the observed phenotype are unlikely to lead to the identification of residues specifically involved in secretion.

pDW306, although not possessing a full size recognisable product, appeared to have a band at approximately 35KDa, the predicted size of the catalytic domain, which is not present in any other samples, and therefore was assumed not to be non-specific background. This was investigated further by cloning the 2Kb *Sph*I/*Eco*RI *celV*-containing fragment of pDW306 into pT7-6 (figure 4.1), and overexpressing in the T7 expression system, as used during the purification of CelV (section 2.9.4). As figure 8.2 shows, Western analysis of resultant periplasmic fractions from various time points of the experiment confirmed the presence of such a product. The figure shows a comparison of the mutant forms of CelV from DW306 and HA10. At each time point, cellulase activity was comparable, but, as the Western shows, the intensity of bands recognised by the antibodies varies considerably.

8.3 Investigation of the effect of mutant derivatives of CelV on the secretion of pectate lyase

The cell fractions isolated in order to assay cellulase activity of the eleven mutants were also investigated with regard to pectate lyase activity (section 2.2.6), the results of which are shown in figure 8.3, and an example of the well assays used in figure 8.4.

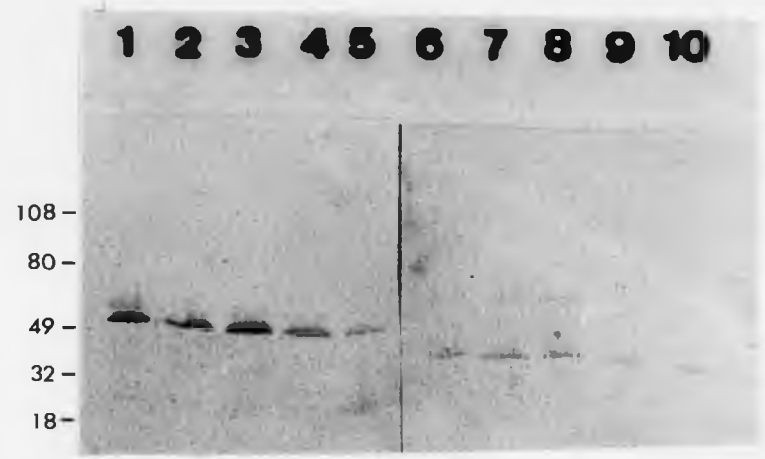


Figure 8.2 T7 expression of mutant cellulases.

Mutant derivatives of *celV* from pDW306 and pHA10 were overexpressed in the T7 system (section 2.9.4). Samples were taken at various time points following induction, and a periplasmic fraction taken (2.2.4) and subjected to Western analysis (sections 2.11.4-2.11.6) using anti-CelV polyclonals.

Lanes:	1 = 30'		6 = 30'		
	2 = 60'		7 = 60'		
	3 = 120'	HA10	8 = 120'		DW306
	4 = 180'		9 = 180'		
	5 = 240'		10 = 240'		

Positions of molecular weight standards are shown in kDa.

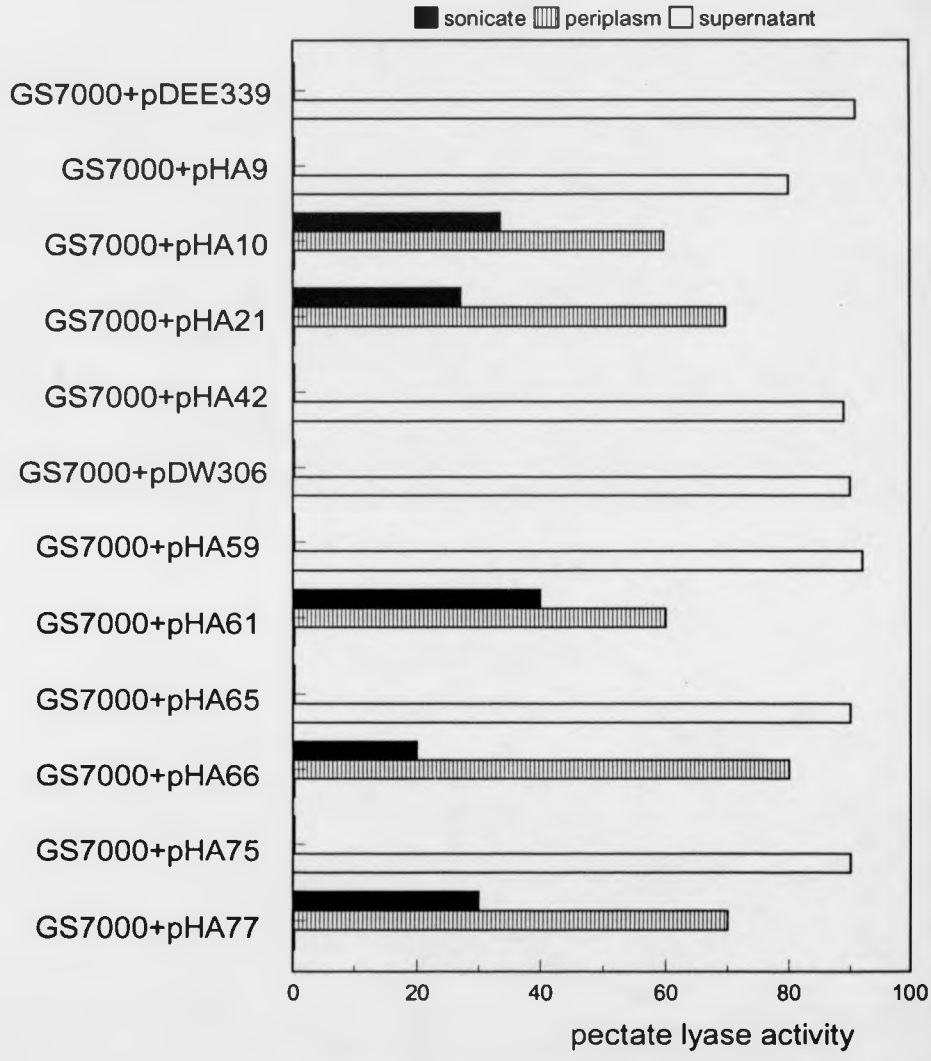


Figure 8.3 Pectate lyase assays of secretion mutants.

GS7000 + pDEE339 is compared with mutant derivatives. Pectate lyase activity of sonicate, periplasm and supernatant fractions is shown, expressed as percentage SCRI193 total activity, based on dilutions of whole culture sonicate of SCRI193. Fractionation control: see legend to figure 7.4.

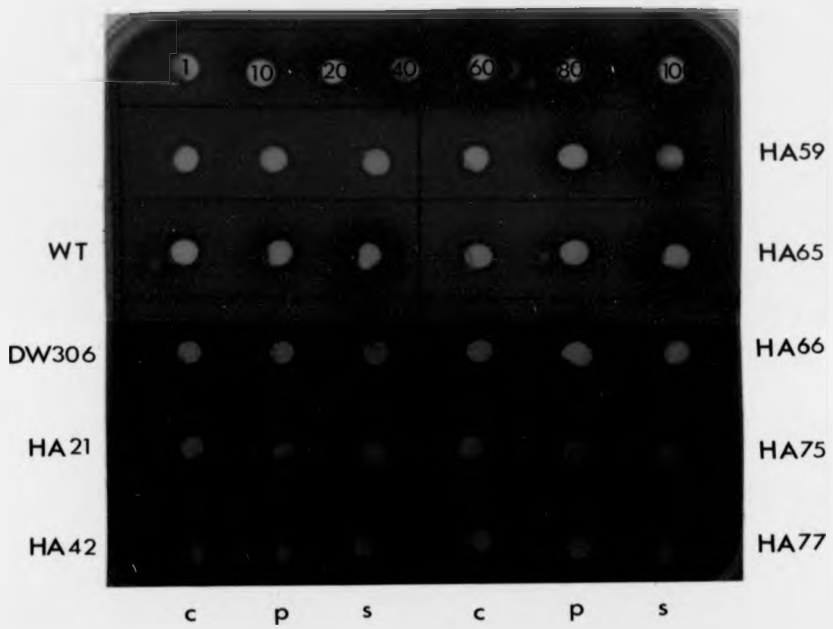


Figure 8.4 Pel well assays of secretion mutants.

An example of one of the Pel well assay plates used to calculate pectinase activity of cell fractions from secretion mutants, as described in figure 8.3. The top row shows a series of dilutions of SCRI193 whole culture sonicate, from 1% to 100%, as marked in the centre of each inoculation site. For the remainder, strains are given on either side, and cell fractions are indicated below the photograph. WT denotes GS7000 + pDEE339; S = supernatant; P = periplasm; C = cytoplasm.

As the data show, several of the mutants deficient in the secretion of CelV, by virtue of a mutation within the cellulase gene, are also severely deficient in the secretion of pectate lyase, while the remainder are completely unaffected. This was investigated further by Western analysis of the samples prepared for Anti-CelV Western analysis, using monoclonal antibodies against PelD and endo-polygalacturonase (Peh) (section 2.12.6). An example from each group of mutants, those deficient in pectate lyase secretion and those unaffected, was investigated, along with the wild type system, GS7000+pDEE339, in particular to verify that the effect observed was not due to degradation of pectate lyase, and also to investigate whether the same effect was seen for polygalacturonase, another of the enzymes secreted by the Out apparatus. As figure 8.5 shows, the results given by Western analysis directly support those given by enzymatic analysis. Both Peh and PelD are as efficiently secreted by HA9 as by GS7000+pDEE339; while neither are secreted at all, within the levels of detection, by HA10. In neither case is there any evidence for degradation of the enzyme concerned.

Comparison of the anti-CelV Western data and the pectate lyase data shows that all of the mutants which exhibit a full size, stable product accumulated in the periplasm (HA10, HA21, HA61, HA66 and HA77) block the secretion of pectate lyase. Conversely, all of the mutants for which a full size, stable product was not detected by the antibodies

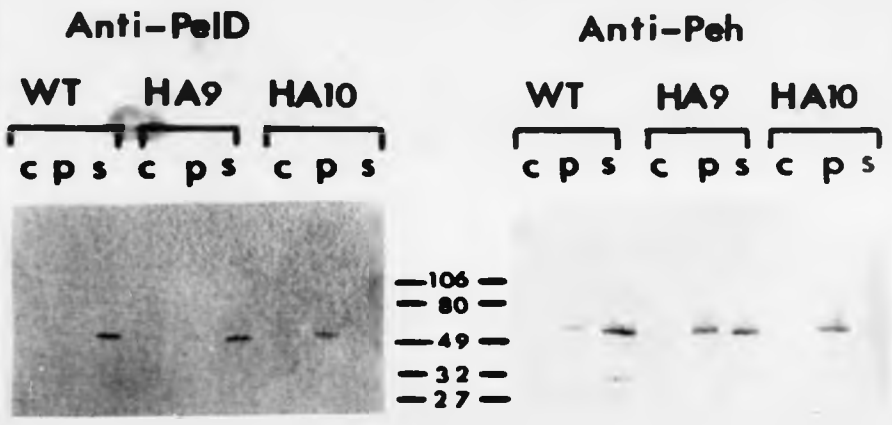


Figure 8.5 Western analysis of secretion mutants, using anti-pectinase antibodies.

Western analysis of a representative mutant from each class, compared with the wild type, GS7000 + pDEE339 (denoted as WT). Strains are shown across the top. C = cytoplasm; P = periplasm; S = supernatant. The gel on the left was blotted using monoclonal antibodies against PelD, the one on the right using monoclonal antibodies against endo poly galacturonase (Peh). Positions of molecular weight standards are shown in the centre, in kDa.

used continue to be able to secrete pectate lyase and polygalacturonase with the same efficiency as the wild type.

8.4 Investigation of the effect of mutant derivatives of CelV on the secretion of wild type CelV

The effect on pectate lyase secretion observed for several of the mutants isolated lead to the question as to whether these mutant forms of CelV would have the same effect on the secretion of wild type CelV. To answer this question, pHA10 and pHA21 were introduced into the wild type strain HC131 by electroporation (section 2.5.2). The resulting strains were grown in LB and fractionated (section 2.2.4) into cytoplasmic, periplasmic and supernatant fractions. Supernatant and cytoplasmic fractions were concentrated and all fractions were used to prepare samples for Western analysis (section 2.11.4). These were subjected to Phast gel SDS PAGE, and Western analysis using anti-CelV antiserum. (sections 2.11.5 and 2.11.6). The results, for HC131 compared with HC131 containing pHA10 or pHA21, are shown in figure 8.6.

As the gel shows, both HC131 + pHA10 and HC131 + pHA21 exhibit a degree of secretion of CelV. In both cases, a greater proportion of CelV is located in the periplasm, presumably representing the mutant form plus a proportion of the wild type form. A significant proportion, presumably the wild type form, is, however, secreted to the supernatant fraction. This indicates that the mutant forms of CelV that are capable of interfering with the secretion of pectinases

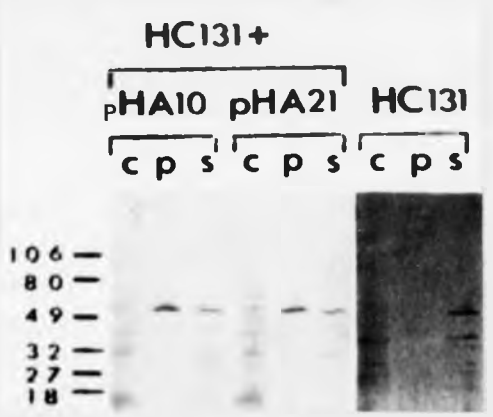


Figure 8.6 Western analysis of HC131 expressing mutant derivatives of *celV*.

Western analysis was performed using polyclonals against CelV. Strains are shown at the top. S = supernatant; P = periplasm; C = cytoplasm. Positions of molecular weight standards are shown on the left, in kDa.

do not have the same effect on secretion of wild type cellulase, but do nevertheless significantly reduce the efficiency of its secretion.

8.5 Sequence analysis of mutant forms of the cellulase gene

For the purpose of sequencing, the 2Kb *Sph*1/*Eco*R1 fragments containing the mutant forms of *celV* were cloned into pUC19 cut with the same two enzymes (see figure 6.3), resulting in plasmids called pUCV10, pUCV21, pUCV306, and so on, derived from the original mutant names. Each plasmid was introduced into TG1, and plasmids prepared using either Qiagen Midi kits (section 2.3.3) or Promega Magic Miniprep kits (section 2.3.2), depending on the quantity of plasmid required. Single stranded sequencing template was prepared (section 2.4.7), and sequenced (sections 2.4.8 and 2.4.9) using a series of 17mer oligo-nucleotide primers (table 2.4) that were designed to anneal at intervals along the sequence of *celV* (appendix 1). Mutant-derived plasmids were sequenced alongside pUCV-1, the wild type, to allow the identification of mutations.

The mutant derived from PCR mutagenesis possesses a deletion of eleven base pairs, from position 1568 to 1578, as illustrated in figure 8.7. Such a deletion could be explained as an error by Taq Polymerase, based on the fact that the area surrounding this deletion contains several repeat patterns. The region concerning the deletion contains an imperfect tandem repeat, GCAATATC and GCAATGTG, and there are several similar sequences in the surrounding region. The

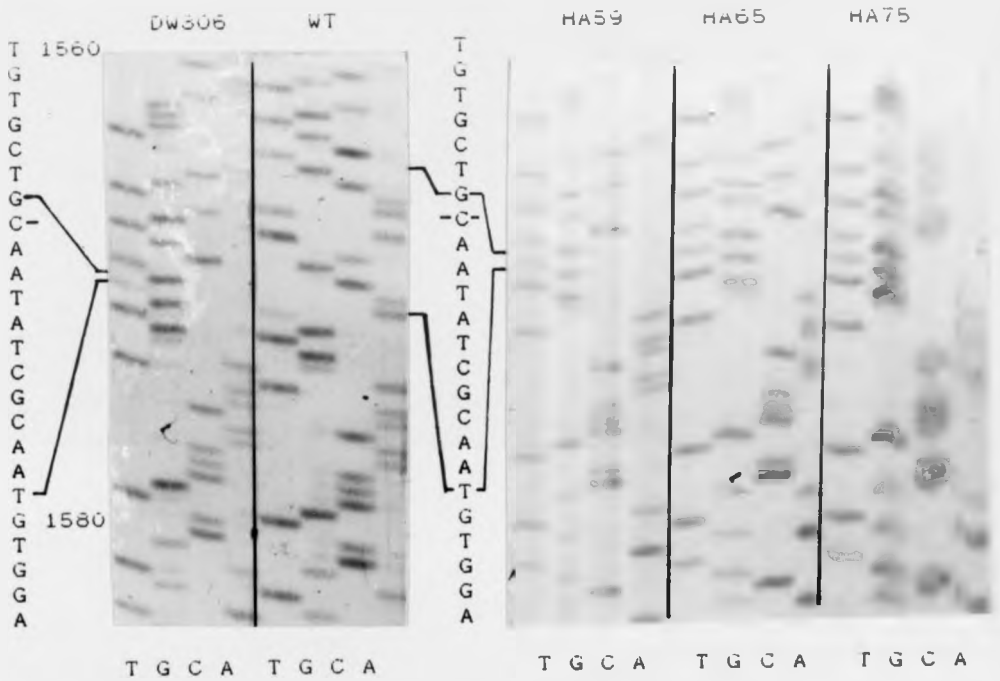


Figure 8.7 Sequence analysis of Dw306, HA59, HA65 and HA75.

The nucleotide sequences of *celV* derivatives from DW306, HA59, HA65 and HA75 are shown, compared with the wild type.

The nucleotide sequence of the region around the deletion is shown parallel to the photographs, and the position of the 11bp deletion is indicated in bold.

same deletion, however, occurs in 3 of the mutants isolated from hydroxylamine mutagenesis, HA59, HS65 and HA75, as figure 8.7 shows. HA59 and HA65 were isolated from the same mutagenesis experiment. Therefore these 4 mutants represent 3 independent isolates of the same deletion. It seems, therefore, that this precise eleven base pair deletion is a spontaneously occurring phenomenon, independent of the mutagenesis procedure, which is simply picked out by the screening procedure used. Such a phenomenon would be easier to explain if the deletion was of 9bp, the size of one of the imperfect tandem repeats. Deletions of this type have been previously observed by McGowan (1992), although in both of the cases described the region deleted was a whole (imperfect) repeat. He explained this phenomenon by adapting a model proposed by Efstratiadis *et al.* (1980). The idea was that during replication, as the replication fork passed through the region, a mis-match of repeats (where the downstream repeat of, say, the top strand pairs with the upstream repeat of the the lower strand) means that one of the downstream repeats no longer has a complementary sequence, and therefore loops out, resulting in its absence in one of the daughter strands. It is more difficult to explain why the deletion is of 11bp, rather than 9bp. However, there are many complex repeat patterns in this region which could be involved in a similar mechanism. The original model by Efstratiadis *et al* (1980) concerned the role that short direct repeats flanking β globin genes are thought to play in deletion of entire genes, where mis-pairing of repeats in a similar way to that described results in looping out of the gene in question on one

strand. In a similar way, short repeats surrounding the 11bp deletion site described here could be important.

Figure 8.8 shows the effect that this deletion has on the amino acid sequence of CelV. As the diagram shows, the frameshift results in abrupt termination of the amino acid sequence. This region is just downstream of the proline, threonine rich linker, so termination at this point results in a truncated protein in which the catalytic domain and linker are (based on the independence of domain functions universally demonstrated for cellulases) intact. This explains why the resulting derivative of CelV remains catalytically active but is not capable of being secreted and is not strongly recognised by the anti-CelV polyclonal antibodies. This coincides with the observation of a product of approximately 30kDa seen in figure 8.2, when the mutant derivative of *celV* from pDW306 was overexpressed in the T7 system. A similar product would be expected if *celV* from HA59, HA65 or HA75 were overexpressed at a high level.

As figure 8.9 shows, both HA9 and HA42, which were isolated from separate mutagenesis experiments, exhibit the same mutation, a G to A transition that results, as figure 8.8 shows, in replacement of glycine by serine. This is approximately mid-way between H98 and E136, two of the most crucial residues predicted to form the active site (Py *et al.*, 1991b). Since this does not have a significant effect on catalytic activity, it seems unlikely that the mutation has a profound effect on the catalytic domain. There are

HA59, HA65, HA75 and DW306

```
1560       1570       1580       1590
..GTCGTGCTGCAATATCGCAATGTGGATAACAACCCG....
   V  V  L  Q  Y  R  N  V  D  N  N  P

..GTCGTGCTGTGTGGATAACAACCCG....
   V  V  L  C  G  *
```

HA9 and HA42

```
950        960        970
..CTGTATGGCAGTTCGCCGAAC....
   L  Y  G  S  S  P  N

..CTGTATAGCAGTTCGCCGAAC....
   L  Y  S  S  S  P  N
```

HA42

```
 1990       2000       2010
..GTATGGGGTGTGGAGCCHTAATCG....
   V  W  G  V  E  P  *

..GTATGGGATGTGGAGCCGTAATCG....
   V  W  D  V  E  P  *
```

Figure 8.8 Mutations identified from sequencing of secretion deficient mutants.

The figure shows the DNA sequence of the three types of mutation observed, together with the effect that these have on amino acid sequence. * indicates a termination codon. DNA sequence positions are given above the sequence, the last digit of each being above the sequence position to which they refer. Nucleotides which undergo transition or deletion are underlined. Refer to appendix 1 for the full wild type nucleotide sequence of *ceIV*.

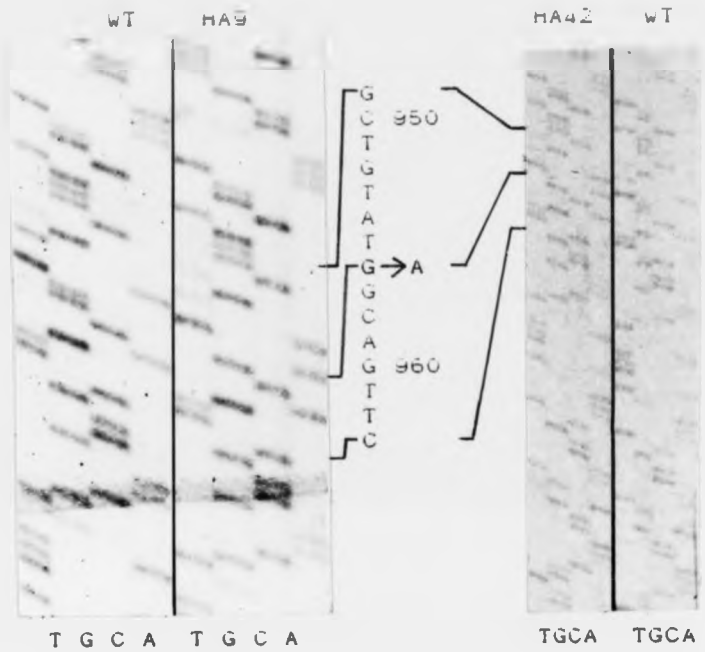


Figure 8.9 Sequence analysis of HA9 and HA42.

The sequences of *celV* derivatives from mutants HA9 and HA42 are shown, together with the wild type for comparison.

The nucleotide sequence of the relevant region is given in the centre, and the position of the G to A transition indicated in bold. These mutations were present in both DNA strands (data not shown).

therefore several possible explanations that remain. The residue concerned could play a role in the interactions between the two functional domains to give a particular global conformation. The mutation might therefore result in a stable but secretion-incompetent conformation, or in a decrease in stability that results in proteolytic liberation of the catalytic domain. Alternatively, the residue could be crucial to recognition of CelV by a component of the secretory apparatus (an Out protein?), and failure to be recognised could mean that the protein becomes susceptible to cleavage by periplasmic proteases, releasing the catalytic domain that is active but secretion-incompetent. These speculative explanations could be clarified if more powerful antibodies were generated that recognised truncated forms of the protein, allowing more precise characterisation of the product which accumulates in the periplasm.

HA42 also possesses a second G to A transition at position 1995, as figure 8.10 shows. As figure 8.8 illustrates, this affects the fourth residue from the carboxy terminus of CelV, replacing glycine with aspartate. To establish which of the two mutations was responsible for the observed phenotype, the mutant derivative of *celV* was subcloned in two halves. The *Sph*I/*Eco*RV 1.1Kb fragment and *Eco*RV/*Eco*RI 0.9Kb fragment (see appendix 1) were ligated (section 2.3.9) into wild type pDEE339 (figure 6.1) digested (section 2.3.4) with the same enzymes, and the two resulting plasmids introduced into GS700o by electroporation (section 2.5.2). The resulting strains were characterised with respect to localisation of cellulase activity (sections 2.2.4 and

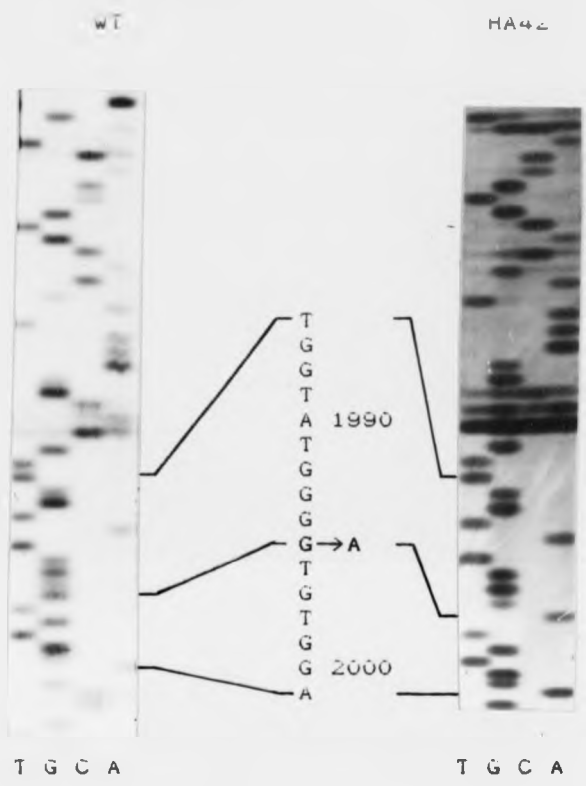


Figure 6.10 Sequence analysis of HA42.

The sequence of the *cel1* derivative of HA42 is compared with the wild type gene, showing the region around the second mutation.

The nucleotide sequence is given at the side, and the position of the G to A transition indicated in bold. The mutation was present in both DNA strands (data not shown).

2.2.5). This established that the transition at position 1995 has no effect on secretion or catalytic activity of CelV, therefore attributing the phenotype to the mutation at position 956, which is common to HA42 and HA9.

Unfortunately, the genetic basis of the other class of mutants, those which interfere in the secretion of pectate lyases, has not, as yet, been established.

8.6 Discussion

The eleven mutants defective in the secretion of CelV isolated can be divided into two distinct classes. The first class have a full size, stable product, which is recognised by the antibodies used in Western analysis, is fully catalytically active, and which in some way interferes in the secretion of the other enzymes secreted by the Out apparatus, pectate lyase and polygalacturonase. The other class have products which are catalytically fully active, but are not full size, and do not have any effect on the secretion of other enzymes secreted by the Out apparatus.

On the basis of the phenotypes isolated, it appears that the mutants isolated can be divided with regard to the stage in the secretion process affected. Since the first class affects the secretion of other enzymes, it seems that these are blocked in the process of translocation, within the Out apparatus itself, thereby in some way blocking the passage of the other enzymes. The other class, though, do not affect the secretion of other enzymes, so that it seems likely that

they are deficient at the level of recognition for secretion. Failure to be recognised for secretion means that they fail to actually enter the translocation pathway of the Out apparatus, and therefore cannot affect the other enzymes.

The fact that the first class of mutants block secretion of pectate lyases to a greater degree than secretion of wild type cellulase is puzzling. Following on from the prediction that these mutants are deficient at the level of translocation itself, one explanation is that wild type CelV has a greater affinity for the recognition component of secretion than the mutant form, thereby reducing the efficiency with which it can enter the translocation stage at which it becomes blocked.

That mutants often possess the same mutation, and that several of the mutations identified seem to be spontaneous, all of the same nature, together with the low level of secretion mutants identified compared with other classes, all suggest that these are indeed rare. It perhaps even suggests that the precise class of point mutant pursued, those which would identify a recognition motif for secretion, may not exist. This could infer that recognition for secretion requires general conformational information that is to a certain extent flexible, and not affected by small sequence changes. Alternatively, it suggests that conformation is strongly dependent on residues that have a secretory significance, so that mutation of these always affects stability of the protein.

CHAPTER 9

ISOLATION OF SUPPRESSORS

9.1 Introduction

Following the isolation of mutants of a given phenotype, the logical next step is the isolation of suppressors in order to use the original mutants to extend the information available. There are two approaches which can be taken, the isolation of intragenic suppressors, within the gene originally mutated, and the isolation of extragenic suppressors, within genes encoding proteins that interact in some way with the original one.

The isolation of intragenic suppressors, i.e. ones within the original gene which to a greater or lesser extent reverse the phenotype of the mutant, would be useful in this context in that they would hopefully extend the information that we have about the requirements of secretion: and, particularly, about the nature of interactions between sites in the protein.

The isolation of extragenic suppressors is in this context particularly important with regard to the Out apparatus. Although some clues are emerging about the functions of several of the Out proteins, such as OutD, OutE and OutO, nothing is known about the way in which the secreted proteins interact with the Out apparatus. This is largely because the nature in which the Out proteins interact to form a complex means that it is difficult to investigate individual proteins. In Ecc this problem is compounded further by the fact that the cluster of genes so far isolated does not form a reconstituted system when expressed

from a plasmid in *E. coli*. There are also apparent problems with using marker exchange onto the chromosome in SCRI193. These factors severely compromise the amenability of the Ecc Out apparatus to detailed study. The use of extragenic suppressors could be an important way to start investigating this problem, because it would, theoretically, result in the identification of components of the Out apparatus which interact in some way with CelV.

9.2 Isolation of intragenic suppressors

DNA from pDEE339 mutant derivatives pHA9, pHA10, pHA21, pHA42 and pDW306 was prepared (section 2.3.2) and then subjected to a further round of the hydroxylamine mutagenesis treatment (section 2.8.1), which lead to the isolation of all except pDW306. Following dialysis and "Genecleaning" (section 2.3.7), the resulting DNA was used to transform GS7000 by electroporation (section 2.5.2). Transformants were selected on LB, kanamycin plates, and then picked onto cellulase assay plates (section 2.2.1), to allow the identification of any transformants exhibiting an increase in halo size. For mutants of the class which are deficient in Pel secretion, transformants were also screened for increased halo size on Pel assay plates (section 2.2.3). Such isolates were then investigated further, by cell fractionation into supernatant, periplasm and cytoplasm samples (section 2.2.4), followed by assays of cellulolytic activity of the cell fractions. Table 9.1 shows the number of transformants screened for each of the mutants investigated, and the resulting frequencies of suppressors.

Mutant	Transformants screened	Suppressors isolated	Frequency of suppressors
DW306	2600	-	0
HA42	2240	MD42A	0.13%
		MD42B	
		MD42C	
HA10	1600	MD10J	0.06%
HA21	2450	MD21F	0.08%
		MD21M	

Table 9.1 Screening for intragenic suppressors.

Mutant pDEE339 derivatives from the mutants listed in the first column were subjected to hydroxylamine mutagenesis for 36 hours (section 2.8.1), and resulting transformants screened on CMC plates for restoration of cellulase secretion. The table shows, from left to right, the mutants used; the number of transformants screened; intragenic suppressors isolated; and the frequency at which these occurred.

A total of six suppressors were isolated.

9.3 Characterisation of intragenic suppressors

Strains were grown to an optical density of A_{600} = 1.2-1.4 and fractionated into supernatant, periplasmic and cytoplasmic fractions (section 2.2.4). These were then subjected to cellulase activity assays (section 2.2.5), as shown in figure 9.1. The same fractions were also subjected to pectate lyase activity assays (section 2.2.6), the results for which are shown in figure 9.3. Supernatant fractions were concentrated by TCA precipitation (section 2.11.4) and these and periplasmic fractions were subjected to Western analysis using anti-CelV polyclonal antibodies, the results of which are shown in figure 9.2.

As figure 9.3 demonstrates, the three suppressors isolated from mutants of the class that are deficient in Pel secretion, MD10J, MD21F and MD21M, exhibit a high level of restoration of their ability to secrete pectate lyase (as compared with the parent mutants, shown in figure 8.3). With regard to cellulase secretion, however, they have varied phenotypes. As figure 9.1 shows, MD10J shows periplasmic accumulation of cellulase, while MD21F and MD21M exhibit at least some degree of secretion, although not to wild type efficiency. Consultation of figure 9.2, showing Western analysis of the suppressors, provides an important clue as to why MD10J has a different phenotype. Whereas the original mutant, HA10, exhibited accumulation of a full size stable product in the periplasm (like HA21, which is shown in

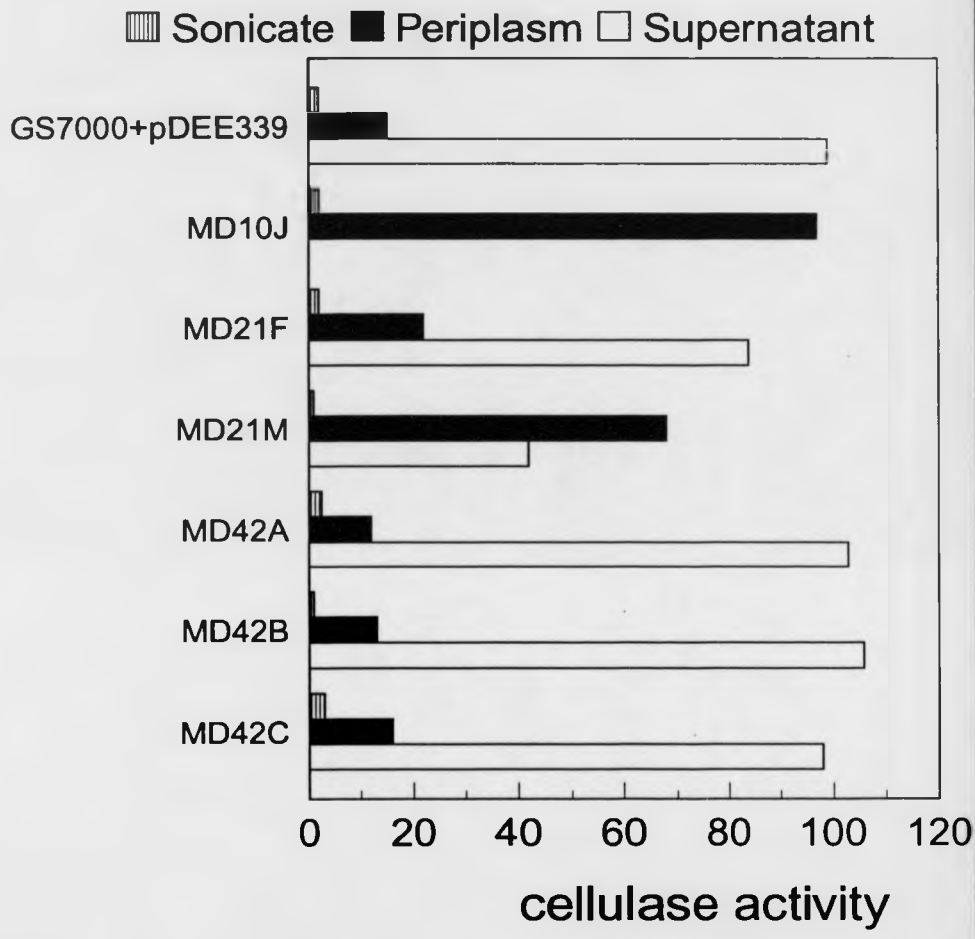


Figure 9.1 Cellulase assays of intragenic suppressors.

Sonicate, periplasm and supernatant fractions (section 2.2.4) of the strains indicated were assayed (section 2.2.5). Results are expressed as percentage SCRI193 total activity, based on a standard curve constructed using serial dilutions of SCRI193 whole culture sonicate.

Fractionation control: at least 92% *B* galactosidase activity is located in sonicate fractions.

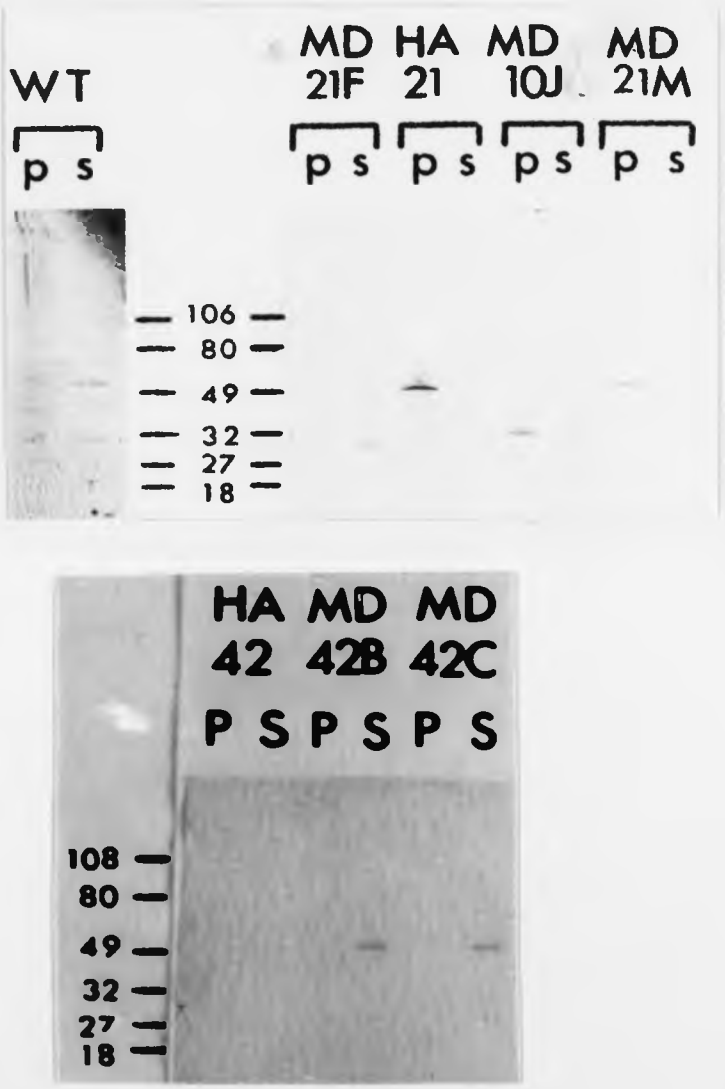


Figure 9.2 Western analysis of intragenic suppressors.

The diagram shows anti-CelV Western analysis of the suppressors isolated, compared with their parent mutants. Strains are shown at the top. S = supernatant; P = periplasm; C = cytoplasm. GS7000 + pDEE339 is designated by WT. Molecular weight standards are shown in KDa.

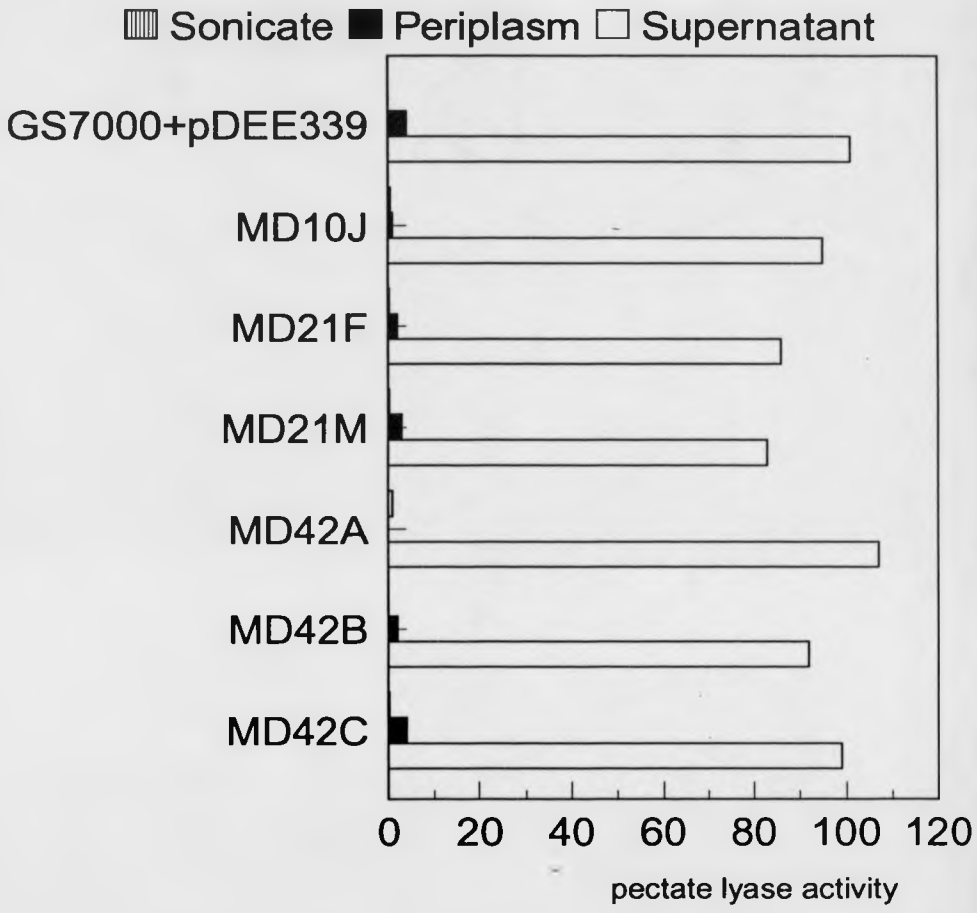


Figure 9.3 Pectate lyase assays of intragenic suppressors.

The graph shows the results of pectate lyase activity assays (section 2.2.6) of sonicate, periplasm and supernatant fractions (section 2.2.4) of the strains indicated. Results are expressed as percentage SCRI193 total activity, based on a standard curve drawn from serial dilutions of SCRI193 whole culture sonicate.

Fractionation control: at least 92% *B* galactosidase activity is located in the sonicate fraction.

figure 9.2 for comparison), the suppressor MD10J has no such product. Instead, a periplasmic band is seen at approximately 32KDa (perhaps representing the catalytic domain), which is reminiscent of the results seen for the other class of mutants isolated. The results presented in chapter 8 lead to the prediction that the class of mutants which block Pel secretion are blocked in the translocation machinery itself, while the class of mutants which only affect Cel secretion are blocked at the level of recognition. In other words, it appears that MD10J represents the conversion of the former mutant class to the latter, as a result of a mutation which truncates the product or affects stability, so that the catalytically active but secretion-incompetent catalytic domain is proteolytically released. Since such a mutant form of CelV is not recognised for secretion, it remains free in the periplasm, rather than becoming blocked in the translocation apparatus.

Three suppressors were isolated for mutants of the class which do not affect the secretion of pectate lyase, MD42A, MD42B and MD42C, although these were isolated from the same mutagenesis experiment and therefore could be siblings. Figure 9.3 shows that, as expected, these have no effect on the secretion of pectate lyase. Inspection of figures 9.1 and 9.2 indicates that in all three cases suppression is very good, resulting in a full-size, stable product which is secreted to an efficiency approaching that of the wild type, GS7000 + pDEE339.

9.4 Sequence analysis of intragenic suppressors

Since mutants HA10 and HA21 had not been fully characterised in terms of nucleotide sequence, suppressors to these were not sequenced. Others were plasmid sequenced (sections 2.4.6 - 2.4.9) using primers specific to *celV* (table 2.4).

The sequence of the suppressors MD42A, MD42B and MD42C are shown in figure 9.4. The effects that these sequence changes have on amino acid sequences are illustrated in figure 9.5. As the figures illustrate, MD42A, MD42B and MD42C possess the same sequence change, confirming the previous suspicion that they may be siblings. The change is a reversion of the mutation to wild type, an A to G transition, which is not normally expected to result from hydroxylamine mutagenesis.

9.5 Isolation of extragenic suppressors

The search for extragenic suppressors was focused on the *out* cluster. Firstly, because it is the nature of interactions between secreted proteins and the Out machinery which are of particular interest. Secondly, since the *out* cluster has been studied in detail, it is amenable to study. However, since the *out* cluster of Ecc cannot be used to reconstitute secretion in *E. coli*, it should be borne in mind that there may be other, as yet unidentified, factors involved.

The basic strategy used was to specifically mutagenise the *out* cluster and then introduce this into GS7000 expressing one of the pDEE339 derivatives carrying a secretion-deficient mutant form of *celV*, and then to screen on CMC

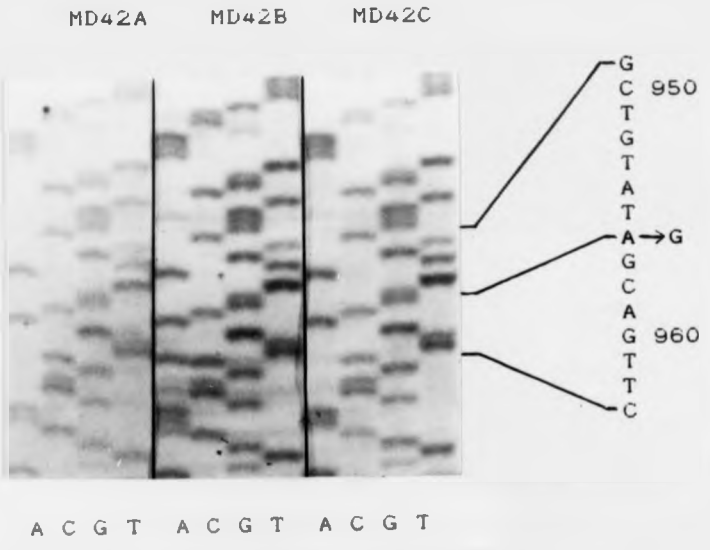


Figure 9.4 Sequence analysis of intragenic suppressors.

The nucleotide sequences of *cel1* derivatives from MD42A, MD42B, and MD42C are shown.

The nucleotide sequence of the relevant region is given at the side of the photograph, and the position of reversion of the mutation seen in MA42 is indicated in bold.

HA42

```
950        960        970
..CTGTATAGCAGTTCGCCGAAC....
   L  Y  S  S  S  P  N
```

MD42A, MD42B and MD42C

```
950        960        970
..CTGTATGGCAGTTCGCCGAAC....
   L  Y  G  S  S  P  N
```

Figure 9.5 Sequence changes identified from analysis of intragenic suppressors.

The figure shows the DNA sequence of HA42 compared with the suppressors MD42A, MD42B and MD42C. DNA sequence positions are given above the sequence, the last digit of the number being above the sequence position to which it refers. The nucleotide which undergoes reversion is underlined.

plates for restoration, to a greater or lesser extent, of secretion of CelV.

Mutagenesis of the *out* cluster was achieved using strain JNH1, constructed by J.N. Housby. This has a Tn5 transposon (encoding kanamycin resistance) linked to the *out* cluster, such that phiKP cotransduction frequencies are between 5 and 57% i.e. when a high titre phiKP lysate of JNH1 was used to transduce a range of Out$^-$ mutants that had been mapped to positions across the whole cluster, the frequency of Out$^+$ transductants varied from 5 to 57%, depending on the mutant transduced (Housby 1993). The generalised transducing phage phiKP (Toth *et al.*, 1993) was used to prepare a high titre lysate of strain JNH1 (section 2.7.1), and the resulting lysate was subjected to hydroxylamine mutagenesis (section 2.8.3). Initially, a killing curve was constructed to determine the time of exposure required to give a three log drop in phage count (pfu/ml). Such a drop indicates a level of mutagenesis that is sufficient to give a detectable level of mutants, but at the same time is adequately low to minimise the likelihood of double mutants.

The mutagenesis reaction mix (section 2.8.3) was set up and incubated at 37°C. Samples were taken every 6 hours for 48 hours, and diluted 100 times in LBSE (table 2.1) and titrated (section 2.7.1) on HC131 lawns. The information gained was used to construct a graph of time of exposure against phage count, as figure 9.6 shows. A control experiment was carried out in parallel, where hydroxylamine

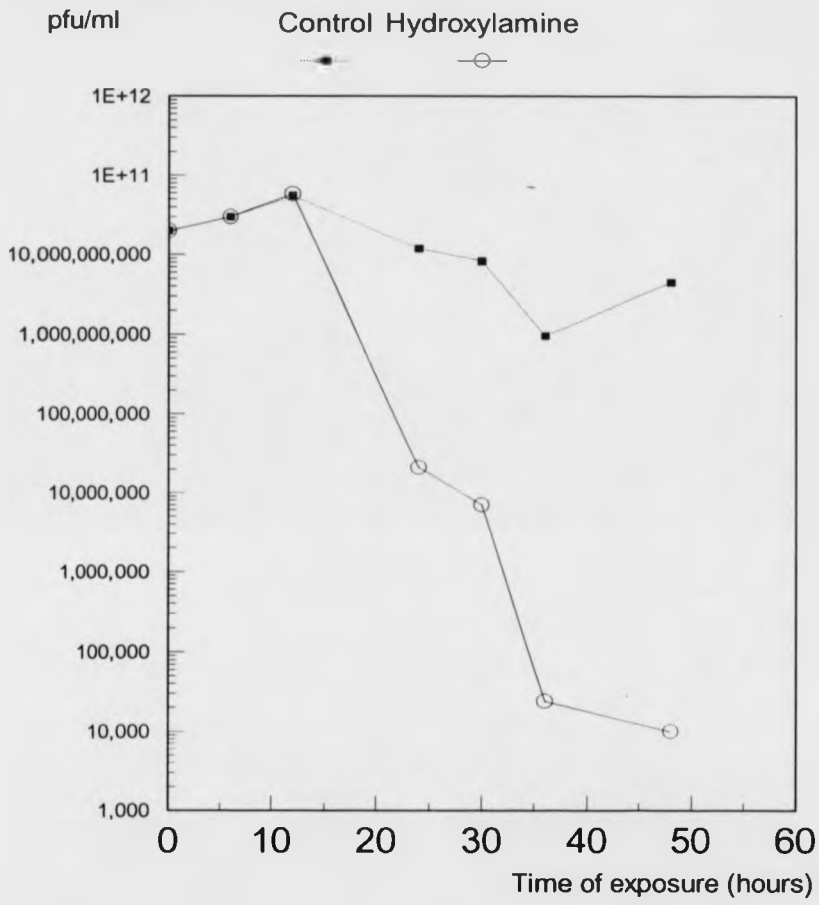


Figure 9.6 Hydroxylamine killing curve for phiKP.JNH1

The graph shows the time of exposure in hours against the plaque forming units per millilitre, observed when the treated lysate was used to infect HC131. Treatments (section 2.8.3) are indicated at the top of the graph. Control indicates that hydroxylamine solution was substituted with water.

solution was replaced with water: this gives an indication of the reduction of phage count that is not due to hydroxylamine mutagenesis. Using the information gained from figure 9.6, the lysate was exposed to hydroxylamine for 22 hours, then precipitated (section 2.8.3).

The problem with using strain JNH1 is that the *out* cluster is linked to the kanamycin resistance gene, the same resistance as that encoded on mutant derivatives of pDEE339. The solution to this problem was to interrupt the kanamycin resistance gene of pDEE339 derivatives with a cassette encoding the chloramphenicol resistance gene, derived from the plasmid pDAH330 (D. Hodgson, pers. com.; figure 9.7). This produced plasmids pCM306, pCM9, pCM10 and pCM21, derived from pDW306, pHA9, pHA10 and pHA21, respectively. These were used to electroporate (section 2.5.2) GS7000, selecting for chloramphenicol resistance. The resulting strains were then transduced (section 2.7.2) with mutated phiKP.JNH1, and transductants selected on LB, chloramphenicol, kanamycin plates. Chloramphenicol and kanamycin resistant transductants were then picked onto cellulase assay plates (section 2.2.1).

Unfortunately, although a large number of transductants were screened (2985 for DW306; 1900 for HA9; 2105 for HA42; 600 for HA10) no transductants were isolated which exhibited any increase in halo size on CMC plates. This is not due to inefficiency of the mutagenesis procedure, because when the mutated lysate was used to transduce SCRI193, 0.67% were Out$^-$, indicating a reasonable level of mutagenesis of the

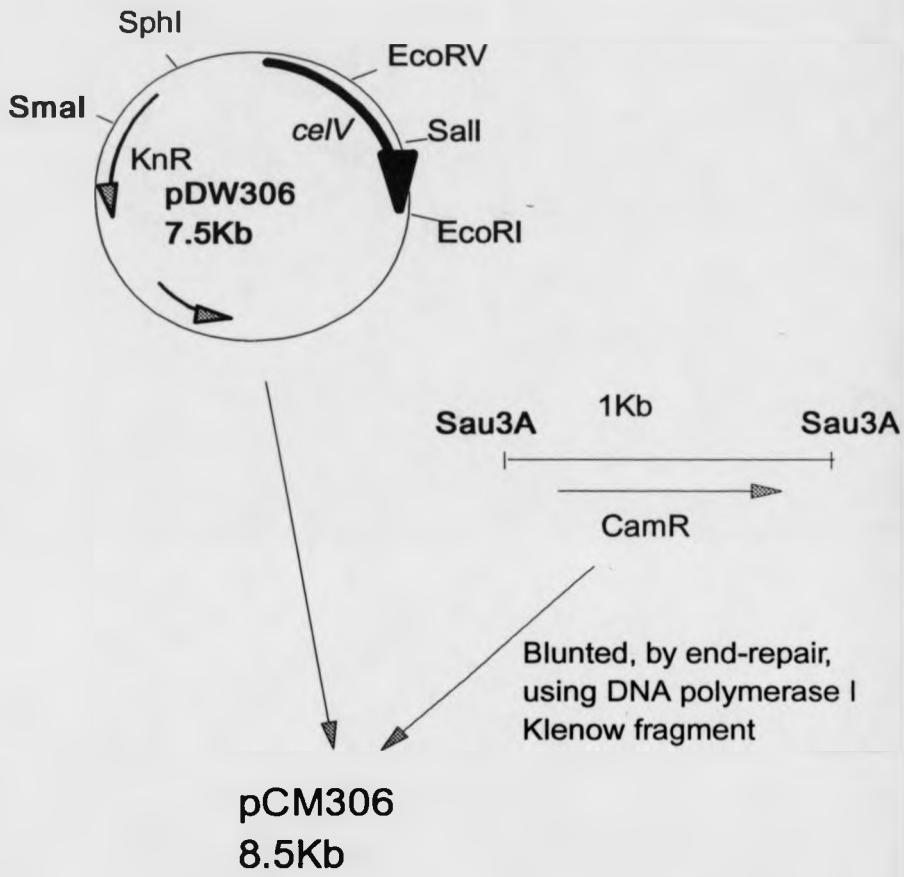


Figure 9.7 Cloning of a chloramphenicol cassette into mutant derivatives of pDEE339.

The figure shows the construction of pCM306 from pDW306, as an example.

out cluster, very similar to that observed by Housby (1993).

9.6 Discussion

Several intragenic suppressors were isolated for mutants of the class that are deficient only in cellulase secretion. These, however, proved to be siblings, and resulted from direct reversion of the original G to A transition. Failure to isolate more interesting suppressors is likely to be the result of the nature of the original mutations, in that they are not easily suppressed by changes elsewhere in the protein. This is probably particularly true of the mutants which contain the 11bp deletion that produces a frameshift that results in introduction of a stop codon 5' of the region encoding the cellulose binding domain. Since a whole series of termination codons would then be present downstream, truncation of the protein could only be prevented by an insertion that restored the original reading frame, and as chapter 10 will show, secretion is dependent on the presence of the whole protein. The practical difficulty of isolating suppressors is compounded by the fact that the range of species of mutation was small, reducing the likelihood of finding one that is amenable to suppression.

Several interesting intragenic suppressors were isolated to mutants of the class that are deficient in Pel secretion. However, further analysis of these is dependent on the characterisation of the primary mutations.

With regard to the search for extragenic suppressors, it appears that the approach chosen here was inappropriate. As with the search for intragenic suppressors, a major contributing factor is likely to be that the phenotypes of the mutants used are such that they cannot easily be suppressed. Continuation of such an approach would therefore require the isolation of a greater range of mutants deficient in the secretion of CelV, if indeed a wider range of such mutants exists.

A more conclusive approach to establishing which of the Out components interact directly with CelV during secretion might be that which was started as described in chapter 6. Overexpression of cellulase in the strain GS7000 + pUCV-1 resulted in considerable accumulation of cellulase within the cellular fractions. This secretion deficiency appeared to be compensated for by expression of the *out* cluster on cHIL251-3. A useful approach might therefore be to isolate clones of individual *out* genes and test these in the same way.

CHAPTER 10

THE EFFECTS OF DELETIONS ON SECRETION OF CELV

10.1 Introduction

The nature of the information required for secretion of EGZ of *Erwinia chrysanthemi* has been substantially investigated by the construction of a whole range of deleted forms of EGZ, the design of which was strongly influenced by knowledge about the domain nature of EGZ (Py, 1992; Py *et al.*, 1993). These included removal of a small carboxy terminal portion of the cellulose binding domain; removal of the entire cellulose binding domain; lengthening or removal of the linker region; and removal of a large internal portion of the catalytic domain. In all cases the result was a protein which remained stable, and in which the unaffected domains remained functionally active, but which was not secreted. The conclusion, therefore, was that secretion information was contained in both domains, that the linker region was perhaps important in holding the two domains in a particular orientation in relation to one another, and that a certain degree of folding occurred in the periplasm.

There are many differences between EGZ and CelV concerning their structure and secretion. The *out* clusters of the two species are different, each possessing genes not found in the other; secretion of EGZ is efficient even when it is expressed from a high copy plasmid while that of CelV becomes overloaded; and EGZ possesses a disulphide bridge in the cellulose binding domain, the formation of which has been demonstrated to be in some way necessary for secretion, while CelV has no disulphide bond. Therefore, it is dangerous to assume that everything that holds true in one

system will necessarily be true in the other. In particular, the lack of a disulphide bridge in the cellulose binding domain of CelV could mean that its structure is of a looser nature, and that requirements for secretion were less constraining. It was therefore decided that several deletions should be constructed in order to clarify that the same conclusions about secretion requirements could be made as for EGZ.

10.2 Construction of deleted derivatives of CelV

Three deleted derivatives of CelV were constructed, all using the basic principle of cutting within *celV* at a suitable restriction site, and creating blunt ends to allow ligation of this to the *Eco*RI site just beyond the 3' end of *celV*.

pVIC626 was digested (section 2.3.4) with *Sph*I and *Eco*RI to yield a 2Kb fragment carrying *celV*, which was separated from the vector fragment by agarose gel electrophoresis (section 2.3.5) and Genecleaned (section 2.3.7). This fragment was then digested with either *Nae*I (eventually yielding CelV2) *Acc*I (eventually yielding CelV3), or *Apa*LI (eventually yielding CelV4). As appendix 1 shows, *celV* possesses a single *Nae*I site around position 1830, which equates to the middle of the cellulose binding domain of CelV. There is a single *Acc*I around position 1445, which corresponds approximately to the end of the catalytic domain and the start of the linker of the protein. The sole *Apa*LI site is around position 1970, very close to the carboxy terminus of the

cellulose binding domain.

Each of the resulting fragments was ligated (section 2.3.9) to pLG339 (figure 6.1), that had been cut with *Sph*I and *Eco*RI and the vector fragment cut from an agarose gel and Genecleaned. Since the cut *Eco*RI site was incompatible with the sites used to create the deletions, these were sticky-end ligations of the *Sph*I sites. The resulting linear fragment was then end-repaired (section 2.3.8), including dNTPs in the reaction, to allow blunt-end ligation (section 2.3.9). The result was deleted derivatives of pDEE339, named pCELV2, pCELV3 and pCELV4. The deletions were confirmed by sequencing (sections 2.4.6 to 2.4.9) over the junctions, using suitable primers specific to *celV*. The results are shown in figure 10.1.

10.3 Investigation of the effects on secretion

pCELV2, pCelV3 and pCELV4 were introduced into GS7000 by electroporation. The resulting strains were grown in LB and fractionated into supernatant, periplasm and cytoplasm samples (section 2.2.4). Fractions were subjected to cellulase assays (section 2.2.5), the results for which are shown in figure 10.2. Supernatant fractions were concentrated using TCA precipitation (section 2.11.4) and these and periplasmic fractions were used to prepare samples for SDS PAGE (section 2.11.4). They were then subjected to Western analysis as described in sections 2.11.5 and 2.11.6, using polyclonal antibodies against CelV. The results are shown in figure 10.3.

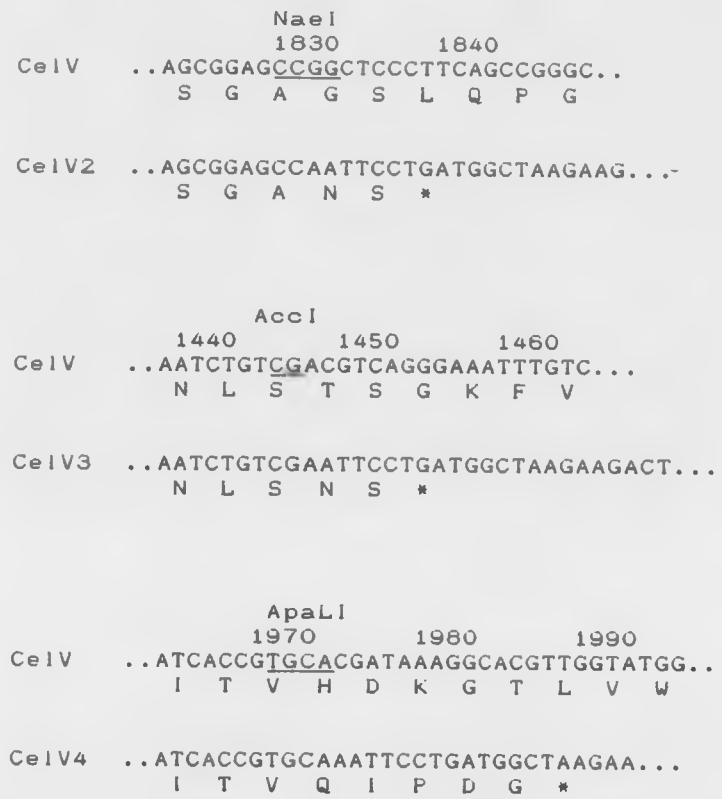


Figure 10.1 Sequence of deletion junctions.

The diagram shows the nucleotide sequence of the regions surrounding the junction between the 3' end of the deleted derivatives of *celV* constructed and the vector, compared with the wild tupe sequence in that region. Below each is given the corresponding amino acid sequence. * denotes a termination codon. Restriction endonuclease sites used in construction of deletions are underlined. Nucleotide numbers indicate the position in the 2Kb fragment whose sequence is shown in appendix 1, and refer to the nucleotide below the last digit of the number.

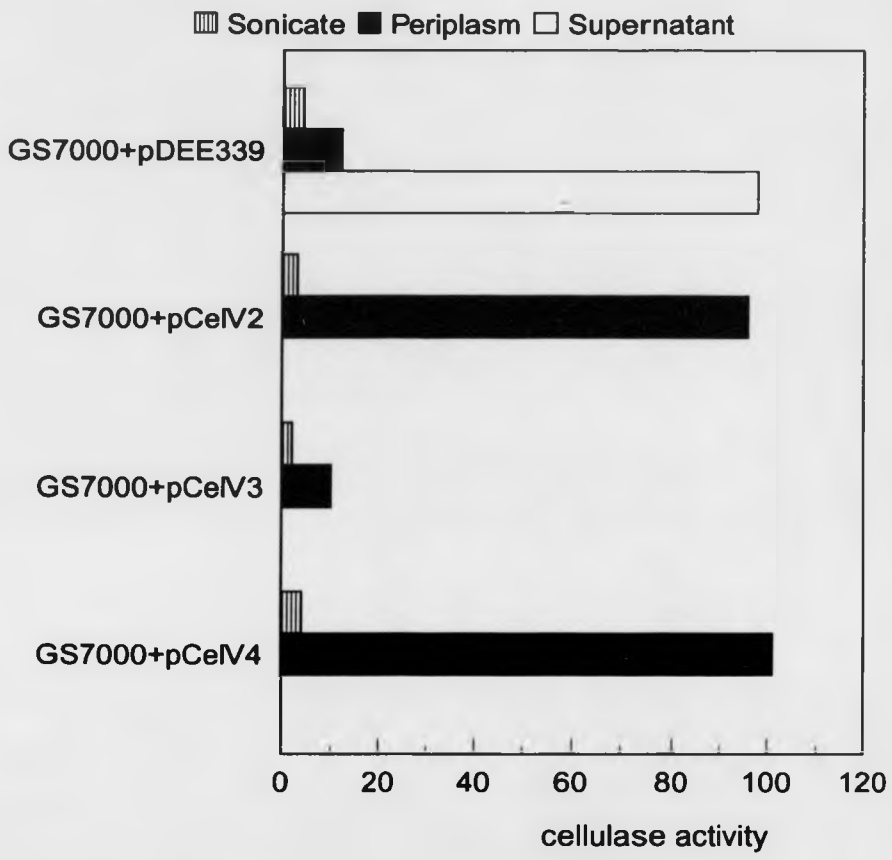


Figure 10.2 Cellulase assays of deleted derivatives of CelV.

The graph shows the results of assays (section 2.2.5) of supernatant, periplasm and sonicate fractions (section 2.2.4) of the strains indicated. Activity is expressed as percentage SCR1193 total activity, based on a standard curve.

Fractionation control: at least 93% *B* galactosidase activity is located in the sonicate fraction.

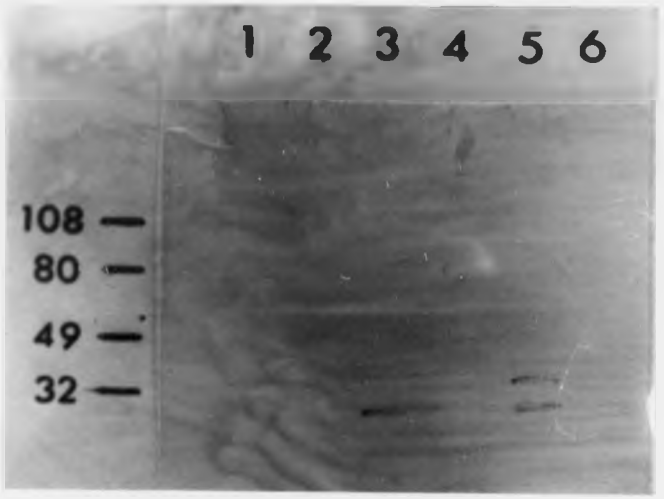


Figure 10.3 Western analysis of deleted derivatives of CelV.

Western analysis using polyclonals against CelV. Molecular weight standards are shown on the left, in KDa.

1 = periplasm) GS7000 + pCelV2
2 = supernatant)

3 = periplasm) GS7000 + pCelV3
4 = supernatant)

5 = periplasm) GS7000 + pCelV4
6 = supernatant)

Figures 10.2 and 10.3 show that both CelV2 and CelV4 both exhibit periplasmic accumulation of a catalytically active derivative of CelV that is not significantly recognised by the anti-CelV polyclonals. In CelV2 quite a considerable section of the CBD is deleted, so the result is not unexpected, if secretion requires information in the CBD. However, in CelV4 only 12 amino acids of the original protein are deleted (from a CBD of approximately 150 amino acids), these being replaced by 5 novel residues. In other words, a very small change to the C-terminus of the protein has a drastic effect on both the conformation of the protein (based on the lack of antigenic product) and on its ability to be secreted. CelV3 has a more significant deletion, which means that catalytic activity is also disrupted.

10.4 Discussion

The results described here indicate that a even a very small deletion at the C-terminus of CelV has a profound effect on the ability of the protein to be secreted. The lack of antigenic product leads us to predict that the reason for this deficiency was the effect that the deletion had on overall conformation. Py *et al.* (1993) reported that many of the deleted forms of EGZ that they constructed showed antigenic products of sizes corresponding to one or the other of the two functional domains, indicating that the deletion results in instability of the protein and proteolytic cleavage of the linker. This is presumably the situation for CelV2 and CelV4, although this could only be

confirmed by the use of more strongly reacting antibodies.

The profound effect of the small deletion of CelV4 indicates that the conformational integrity of the CBD is very highly dependent on sequential integrity. This could be a reflection of the absence of disulphide bridges, and from this point of view it would be interesting to compare the effect with that of a similar deletion of a CBD which possesses a disulphide bridge. The smallest deletion of EGZ is unfortunately 30 residues, a significant portion of the CBD, which (as figure 1.2 illustrated) is much shorter than that of CelV, i.e. only 61 residues.

Before confirming that secretion requires information from both functional domains, as predicted by Py *et al.* (1993) for EGZ, it is important to construct deletions within the catalytic domain that leave the CBD intact.

CHAPTER 11

GENERAL DISCUSSION

11.1 SUMMARY OF RESULTS

In collaboration with other laboratory members (Reeves *et al* 1993), the *out* cluster of genes have been sequenced, revealing extensive homologies to the secretion apparatus of other Gram negative bacteria. This has considerably facilitated further secretion studies.

Production of polyclonal antibodies against CelV, and the establishment of a low copy number plasmid-based expression system enabled secretion deficient mutants to be isolated and characterised, following mutagenesis of *celV*.

A cellulase deficient mutant was used to establish that CelV, although unable to independently cause significant maceration (i.e. when expressed in *E. coli*), plays a crucial role in the synergistic action of the range of exoenzymes during attack of potato tubers.

Hydroxylamine and Polymerase Chain Reaction mutagenesis were used to isolate 11 mutants which retained catalytic activity but were deficient in secretion with the result that cellulase activity accumulated in the periplasmic fraction.

Secretion deficient mutants were assigned to 2 distinct classes. The first class have a full size, stable product that is recognised by the polyclonal antibodies, and that in some way interferes with the secretion of the other enzymes that are secreted by the Out apparatus. The other class exhibit products that are catalytic but are grossly affected

in conformation, so that they are not recognised by the anti-CelV polyclonal antibodies. Mutants of this class fail to have any effect on the secretion of other enzymes.

Deletion of either the entire cellulose binding domain and linker region; or a carboxy-terminal portion of the CBD, even if only 12 amino acids are affected, has a profound effect on both the overall conformation of CelV and its ability to be secreted.

11.2 CONCLUDING REMARKS

Several aspects of the mutagenesis strategy used were very successful. Cloning of *celV* into pLG339 and the expression of the resulting plasmid in GS7000 provided a strain which adequately imitated the wild type, in that CelV was produced at a level low enough to allow efficient secretion; while maintaining the amenity of plasmid-based expression.

The screening procedure used, whereby mutants were first selected for retention of catalytic activity in *E. coli* and then characterised in GS7000, was successful in that it resulted in the isolation of 11 mutants of the required class. However, none of the 11 were found to be *bona fide* recognition-for-secretion deficient mutants, i.e. that were conformationally unaffected and did not interfere in the secretion of other exoenzymes.

The mutants isolated fall into two classes. Those which do not interfere with the secretion of other exoenzymes would

be predicted to be deficient at the level of recognition for translocation across the outer membrane, with the result that they remain free in the periplasm. Either as a result of conformational effects of the mutations responsible, or directly as a result of their failure to be recognised by a component of the secretory apparatus (a periplasmic chaperone?), they are susceptible to periplasmic proteases and degrade into constituent domains. The other class of mutants are conformationally close to wild type, but block secretion of other enzymes. This suggests that they are recognised for secretion as usual, but then become blocked within the translocation machinery itself.

It remains to be seen, therefore, whether it is actually possible to isolate mutants of the type that would identify a discrete recognition motif. The fact that screening of almost 10,000 transformants failed to identify a mutant of this type suggests either that the motif which directs secretion is not dependent on specific residues, instead being of a more general conformational nature; or that residues that are directly involved in recognition by the Out apparatus also play crucial roles in determining global conformation and stability.

One considerable problem with the mutagenesis strategy used is that the fragments of DNA mutagenised were large, so that identification of the mutation responsible was very time consuming. It would therefore have been more appropriate to have mutagenised smaller fragments and then cloned these into the relevant sites of a wild type copy of pDEE339, in a

similar way to the strategy used in PCR mutagenesis. This would make the initial screening steps more complex but would significantly simplify characterisation. However, *celV* does not have many restriction sites that would be suitable for this purpose, and the occurrence of deletions at the restriction site could be a problem.

Several lines of evidence are presented that suggest that cellulase and pectinase initially follow individual pathways (perhaps at the level of recognition or the requirement of chaperones to hold molecules in a secretion-competent state) which converge to a common translocation apparatus. Firstly, the characteristics of the mutant classes isolated suggest that Cel can only interfere with Pel secretion if it is able to reach the translocation step. Secondly, overexpression of Cel does not interfere with the secretion of Pel, even though it significantly reduces the efficiency of Cel secretion. This is despite significant evidence (such as the phenotype of Out mutants) that they are secreted by the same pathway.

11.3 FUTURE WORK

A number of interesting investigations are vindicated by the findings of this work, in order to clarify the conclusions that can be drawn.

With regard to the virulence studies carried out, the role of CelV was clear, but the differing effects of varying cell densities of inocula was an interesting phenomenon. An

important extension of this investigation would be to wash the cells used in the undiluted inocula, to eliminate the effects of high exoenzyme density in the medium. In addition, it would be interesting to extend the study using lower cell densities, and perhaps identify a minimum viable inoculum size that produces a detectable effect.

Efforts to identify interactions with the Out apparatus via isolation of extragenic suppressors of secretion mutants was unsuccessful. However, the apparent ability of the *out* cluster to compensate for the secretion deficiency resulting from overproduction of CelV merits further investigation. Subcloning of the cluster could lead to identification of the rate-limiting step, and since Pel secretion is unaffected, hopefully identify an Out component that interact with CelV early in the secretion mechanism.

The effects of overexpression also merit further investigation, in particular to further characterise the component which is blocked in the sonicate fraction; and to establish whether it is no longer secretion competent.

Both this study and that of B. Py (Py *et al.* 1993; Py 1992) have failed to identify a deletion that remains secretion-competent. Py (1992) also isolated a hybrid between CelV catalytic domain and EGZ CBD, which was not secreted by either species. It would be interesting to extend this approach, by constructing hybrids that possess either one linker (the hybrid constructed by Py *et al* possessed the linkers of each protein in tandem) or a series of different

lengths in the hope that one can assume the appropriate conformation. An additional approach would be to assess whether an intact protein (therefore containing all the information required for secretion) can be used to direct the secretion of a "passenger" domain linked to the carboxy terminus.

11.4 WIDER PERSPECTIVES

The recognition motif for secretion still remains elusive. The main conclusion that can be drawn from investigations is that conformation integrity, of all domains of the protein, is important. The apparent inability to identify specific residues involved in recognition; and the heavy reliance on conformation means that it is difficult to envisage how mutational approaches can be extended.

The requirement for conformational integrity also leads to the question as to how the protein is actually translocated. Other membrane translocation mechanisms (such as mitochondrial and chloroplast import; and Sec-dependent translocation across the inner membrane of Gram negative bacteria) requires that the protein to be translocated is first unfolded.

Many investigations of other membrane translocation apparatuses suffer from a lack of genetic amenability, relying heavily on biochemical investigations, and it is perhaps true that the study of secretion across the Gram negative bacterial outer membrane suffers from the opposite

problem, in that genetics is always the obvious approach.

A major barrier to the use of a biochemical approach is that the Out apparatus consists of a complex that spans both membranes. This eliminates the obvious biochemical approach: to reconstitute the secretory apparatus in outer membrane vescicles and carry out *in vitro* studies using an *in vitro* translated exoenzyme. This would be particularly useful for the study of recognition, which might be expected to involve binding to a receptor on the periplasmic face of the membrane. However, localisation of many of the Out components is based on overexpression, so their locations need to be confirmed.

Another potentially useful approach that cannot be easily applied to this system is the use of cross linking to identify components of the translocation pore. As described in chapter 1, Joly and Wickner (1993) used a preprotein/dihydrofolate reductase fusion, that would jam in the membrane, combined with cross-linking to implicate the involvement of SecA and SecY in translocation across the inner membrane. But to use such an approach to study the outer membrane translocation apparatus would require, firstly, that the protein was translocated normally across the inner membrane, and secondly, that it was capable of being translocated across the outer membrane. A far from simple task.

It is clear therefore, that elucidation of the mechanism by which proteins are specifically secreted by the Out-like

systems of Gram negative bacteria represents a considerable challenge. By contrast, interpretation of the Sec apparatus, which is biochemically more amenable, directed by a simple signal sequence, and has been intensively studied for many years, seems straightforward!

APPENDIX 1

*Sph*I

```
TGCGTAATGCGTTGATTAATCCGCTACATCCCGCGTTAAGCAGCTACGTC 50

TGATTGAGGCGATTCCGGATTTTTACAGTCATAGGCTGTTTAACCCGGTC 100

GGACACTAGGCCTGCTATCGACAGCAATGAGGCTGGCAGGAACCCCATAA 150

TCAGTATGGTAATGAGAAAAATTCTCAACAAAGTGCTTTACAGATGATAC 200

TGATAACTATTATCATCAATTTCGCACTTCGGCAGTAGCTTCTCGCAGGC 250

ACTGAAGGAGAGCACGACATTGCTCACATTGCTTCCAGTATTATTTTAGC 300

CAGCCGACGTGCTGCTTTTTTTTGCCTGAAAAATGACCTCGCCAGATACT 350

TACCTTGCTTTCTATTTTCCTGTAAATAATTAATAAAATCAATATGATTG 400

                    -35                      -10
GGATATTTCCTAAATTGTTTGCGGGAACAAAAATCTCCCTTATCATGCCT 450

ATACCCAAGATCATGTGGGTTCACAATGGAGATAAGGAATATGTGGATGA 500
                                                M  W  M

GAAGGAATCAAATCGTCAGGAAATTGACGTTAGGTGTGGTAACAACGGTG 550
R  R  N  Q  I  V  R  K  L  T  L  G  V  V  T  T  V

CTGGGGATGTCACTCAGTTTTTCAGCATTATCCGCCACGCCAGTAGAAAC 600
 L  G  M  S  L  S  F  S  A  L  S  A  T  P  V  E  T 5
                                   /\

GCATGGTCAGCTGTCCATCGAAAATGGACGACTGGTGGATGAACAGGGGA 650
  H  G  Q  L  S  I  E  N  G  R  L  V  D  E  Q  G   21

AAAGGGTGCAACTGAGAGGGATCAGTTCGCACGGGTTGCAGTGGTTTGGT 700
K  R  V  Q  L  R  G  I  S  S  H  G  L  Q  W  F  G  38
```

Appendix 1 Nucleotide and amino acid sequence of CelV.

The diagram shows the nucleotide sequence of 2kb *Sph*I/*Eco*RI fragment carrying *celV*. The predicted amino acid sequence is shown below. To the right are given numbers of nucleotides, or amino acids of the mature protein. The site of signal sequence cleavage is given by /\. Important restriction sites are given above the sequence.

```
GACTATGTCAATAAAGATTCGATGAAGTGGCTGCGTGATGACTGGGGGAT 750
 D  Y  V  N  K  D  S  M  K  W  L  R  D  D  W  G  I 55

TAACGTATTCCGCGTCGCCATGTACACGGCGGCGGATGGCTATATTTCCA 800
  N  V  F  R  V  A  M  Y  T  A  A  D  G  Y  I  S  71

ACCCCTCCCTCGCCAATAAGGTAAAAGAGGCCGTTGCGGCGGCGCAAAGC 850
N  P  S  L  A  N  K  V  K  E  A  V  A  A  A  Q  S  88

CTCGGTGTTTACATCATCATCGACTGGCATATCTTGTCGGATAACGATCC 900
 L  G  V  Y  I  I  I  D  W  H  I  L  S  D  N  D  P 105

CAATATTTATAAAGCACAGGCAAAAACCTTCTTTGCCGAAATGGCTGGGC 950
  N  I  Y  K  A  Q  A  K  T  F  F  A  E  M  A  G  121

TGTATGGCAGTTCGCCGAACGTGATTTATGAAATCGCCAATGAGCCAAAC 1000
L  Y  G  S  S  P  N  V  I  Y  E  I  A  N  E  P  N  138

GGTGGCGTGACATGGAACGGGCAGATTCGGCCTTATGCGCTGGAAGTGAC 1050
 G  G  V  T  W  N  G  Q  I  R  P  Y  A  L  E  V  T 155

TGACACCATCCGTAGCAAAGATCCCGATAACCTCATCATCGTCGGTACGG 1100
  D  T  I  R  S  K  D  P  D  N  L  I  I  V  G  T  171

                EcoRV
GCACCTGGAGTCAGGATATCCACGACGCAGCGGATAATCAGCTGCCCGAT 1150
G  T  W  S  Q  D  I  H  D  A  A  D  N  Q  L  P  D  188

CCGAATACGATGTACGCGCTGCATTTCTATGCGGGTACGCACGGGCAGTT 1200
 P  N  T  M  Y  A  L  H  F  Y  A  G  T  H  G  Q  F 205

CCTGCGCGACCGCATTGATTATGCACAAAGTCGCGGCGCGGCGATTTTCG 1250
  L  R  D  R  I  D  Y  A  Q  S  R  G  A  A  I  F  221

TCAGCGAATGGGGCACCAGCGATGCGTCCGGCAACGGCGGACCGTTCCTG 1300
V  S  E  W  G  T  S  D  A  S  G  N  G  G  P  F  L  238
```

Appendix 1. continued.

```
CCAGAATCGCAGACCTGGATCGATTTCCTGAATAACCGTGGCGTAAGCTG 1350
  P  E  S  Q  T  W  I  D  F  L  N  N  R  G  V  S  W 255

GGTGAACTGGTCGCTTACCGATAAGTCAGAGGCGTCTGCCGCGCTGGCAC 1400
  V  N  W  S  L  T  D  K  S  E  A  S  A  A  L  A   271

                                                 SalI AccI
CGGGGGCGAGCAAATCAGGCGGCTGGACGGAGCAAAATCTGTCGACGTCA 1450
P  G  A  S  K  S  G  G  W  T  E  Q  N  L  S  T  S  288

GGGAAATTTGTCAGAGAGCAGATTCGTGCAGGTGCGAATCTGGGCGGTGG 1500
  G  K  F  V  R  E  Q  I  R  A  G  A  N  L  G  G  G 305

                                                 AvaI
CGATACGCCGACCACACCAACGGAACCGACCAACCCGGGTAACGGAACAA 1550
  D  T  P  T  T  P  T  E  P  T  N  P  G  N  G  T   321

CGGGTGACGTCGTGCTGCAATATCGCAATGTGGATAACAACCCGTCCGAT 1600
T  G  D  V  V  L  Q  Y  R  N  V  D  N  N  P  S  D  338

GATGCGATTCGTATGGCCGTCAACATCAAAAATACCGGCAGTACGCCGAT 1650
  D  A  I  R  M  A  V  N  I  K  N  T  G  S  T  P  I 355

CAAACTGAGCGATCTGCAAGTACGTTACTACTTCCATGATGATGGCAAAC 1700
  K  L  S  D  L  Q  V  R  Y  Y  F  H  D  D  G  K   371

CTGGCGCGAACCTCTTTGTTGACTGGGCAAACGTCGGCCCTAACAACATC 1750
P  G  A  N  L  F  V  D  W  A  N  V  G  P  N  N  I  388

GTGACCAGCACGGGGACGCCAGCCGCCAGCACTGATAAGGCTAATCGCTA 1800
  V  T  S  T  G  T  P  A  A  S  T  D  K  A  N  R  Y 405

                                                 NaeI
TGTTCTGGTGACCTTCAGCAGCGGAGCCGGCTCCCTTCAGCCGGGCGCGG 1850
  V  L  V  T  F  S  S  G  A  G  S  L  Q  P  G  A   421

AAACCGGTGAAGTGCAGGTGCGTATCCACGCGGGTGACTGGAGCAACGTG 1900
E  T  G  E  V  Q  V  R  I  H  A  G  D  W  S  N  V  438
```

Appendix 1, continued.

*Hae*II

AATGAAACGAATGACTATTCATACGGCGCTAACGTCACGAGCTACGCTAA 1950
N E T N D Y S Y G A N V T S Y A N 455

CTGGGATAAGATCACCGTGCACGATAAAGGCACGTTGGTATGGGGTGTGG 2000
W D K I T V H D K G T L V W G V 471

AGCCGTAATCGTGAGTTGTTAACTTATTAAGCTATTGATTATCGTGCGGG 2050
E P

*Eco*R1
CGTGAATTC

Appendix 1, continued.

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TITLE INVESTIGATION OF THE BASIS OF SPECIFIC SECRETION OF CELLULASE CELV BY THE OUT APPARATUS OF ERWINIA CAROTOVORA SUBSPECIES CAROTOVORA.

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